



**RANDOM PEPTIDES THAT BIND TO GASTRO-INTESTINAL
TRACT (GIT) TRANSPORT RECEPTORS AND RELATED METHODS**

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This application claims priority to U.S. provisional application Serial No. 60/046,595 filed May 15, 1997, which is incorporated by reference herein in its entirety.

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1. INTRODUCTION

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The present invention relates generally to random peptides capable of specific binding to gastro-intestinal tract (GIT) transport receptors. In particular, this invention relates to peptide sequences and motifs, as well as derivatives thereof, which enhance drug delivery and transport through tissue, such as epithelial cells lining the luminal side of the gastro-intestinal tract (GIT). Production of peptides, derivatives and antibodies is also provided. The invention further relates to pharmaceutical compositions, formulations and related methods.

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2. BACKGROUND OF THE INVENTION

2.1. Peptide Libraries

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There have been two different approaches to the construction of random peptide libraries. According to one approach, peptides have been chemically synthesized *in vitro* in several formats. Examples of chemically synthesized libraries can be found in Fodor, S., et al., 1991, *Science* 251: 767-773; Houghten, R., et al., 1991, *Nature* 354: 84-86; and Lam, K., et al., 1991, *Nature* 354: 82-84.

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A second approach to the construction of random peptide libraries has been to use the M13 phage, and, in particular, protein pIII of M13. The viral capsid protein of M13, protein III (pIII), is responsible for infection of

bacteria. Several investigators have determined from mutational analysis that the 406 amino acid long pIII capsid protein has two domains. The C-terminus anchors the protein to the viral coat, while portions of the N-terminus of pIII are essential for interaction with the *E. coli* pillin protein
5 (Crissman, J.W. and Smith, G.P., 1984, *Virology* 132: 445-455). Although the N-terminus of the pIII protein has shown to be necessary for viral infection, the extreme N-terminus of the mature protein does tolerate alterations. In 1985, George Smith published experiments reporting the use of the pIII protein of bacteriophage M13 as an experimental
10 system for expressing a heterologous protein on the viral coat surface (Smith, G.P., 1985, *Science* 228: 1315-1317). It was later recognized, independently by two groups, that the M13 phage pIII gene display system could be a useful one for mapping antibody epitopes (De la Cruz, V., et al., 1988, *J. Biol. Chem.* 263: 4318-4322; Parmley, S.F. and Smith,
15 G.P., 1988, *Gene* 73: 305-318).

Parmley, S.F. and Smith, G.P., 1989, *Adv. Exp. Med. Biol.* 251: 215-218 suggested that short, synthetic DNA segments cloned into the pIII gene might represent a library of epitopes. These authors reasoned that since linear
20 epitopes were often ~6 amino acids in length, it should be possible to use a random recombinant DNA library to express all possible hexapeptides to isolate epitopes that bind to antibodies. Scott, J.K. and Smith, G.P., 1990, *Science* 249: 386-390 describe construction and expression of an "epitope library" of hexapeptides on the surface of M13. Cwirla,
25 S.E., et al., 1990, *Proc. Natl. Acad. Sci. USA* 87: 6378-6382 also described a somewhat similar library of hexapeptides expressed as gene pIII fusions of M13 fd phage. PCT Application WO 91/19818 published December 26, 1991 by Dower and Cwirla describes a similar library of pentameric to octameric random amino acid sequences. Devlin et al., 1990, *Science*, 249: 404-406, describes a peptide library of about
30 15 residues generated using an (NNS) coding scheme for

oligonucleotide synthesis in which S is G or C. Christian and colleagues have described a phage display library, expressing decapeptides (Christian, R.B., et al., 1992, J. Mol. Biol. 227: 711-718).

Other investigators have used other viral capsid proteins for expression of non-viral DNA on the surface of phage particles. For example, the major capsid protein pVIII was so used by Cesareni, G., 1992, FEBS Lett. 307: 66-70. Other bacteriophage than M13 have been used to construct peptide libraries. Four and six amino acid sequences corresponding to different segments of the Plasmodium
10 falciparum major surface antigen have been cloned and expressed in the filamentous bacteriophage fd (Greenwood, J., et al., 1991, J. Mol. Biol. 220: 821-827).

Kay et al., 1993, Gene 128: 59-65 (Kay) discloses a method of constructing peptide libraries that encode peptides of totally random sequence that are longer than those of any
15 prior conventional libraries. The libraries disclosed in Kay encode totally synthetic random peptides of greater than about 20 amino acids in length. Such libraries can be advantageously screened to identify peptides, polypeptides and/or other proteins having binding specificity for a variety of ligands. (See also U.S. Patent No. 5,498,538
20 dated March 12, 1996; and PCT Publication No. WO 94/18318 dated August 18, 1994.)

A comprehensive review of various types of peptide libraries can be found in Gallop et al., 1994, J. Med. Chem. 37:1233-1251.

Screening of peptide libraries has often been done
25 using an antibody as ligand (Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390). In many cases, the aim of the screening is to identify peptides from the library that mimic the epitopes to which the antibodies are directed. Thus, given an available antibody, peptide libraries are excellent
30 sources for identifying epitopes or epitope-like molecules of

that antibody (Yayon et al., 1993, Proc. Natl. Acad. Sci. USA 90:10643-10647).

McCafferty et al., 1990, Nature 348:552-554 used PCR to amplify immunoglobulin variable (V) region genes and cloned those genes into phage expression vectors. The
5 authors suggested that phage libraries of V, diversity (D), and joining (J) regions could be screened with antigen. The phage that bound to antigen could then be mutated in the antigen-binding loops of the antibody genes and rescreened. The process could be repeated several times, ultimately giving rise to phage which bind the antigen strongly.

10 Marks et al., 1991, J. Mol. Biol. 222:581-597 also used PCR to amplify immunoglobulin variable (V) region genes and cloned those genes into phage expression vectors.

Kang et al., 1991, Proc. Natl. Acad. Sci. USA 88:4363-4366 created a phagemid vector that could be used to express the V and constant (C) regions of the heavy and light
15 chains of an antibody specific for an antigen. The heavy and light chain V-C regions were engineered to combine in the periplasm to produce an antibody-like molecule with a functional antigen binding site. Infection of cells harboring this phagemid with helper phage resulted in the incorporation of the antibody-like molecule on the surface of
20 phage that carried the phagemid DNA. This allowed for identification and enrichment of these phage by screening with the antigen. It was suggested that the enriched phage could be subject to mutation and further rounds of screening, leading to the isolation of antibody-like molecules that were capable of even stronger binding to the antigen.

25 Hoogenboom et al., 1991, Nucleic Acids Res. 19:4133-4137 suggested that naive antibody genes might be cloned into phage display libraries. This would be followed by random mutation of the cloned antibody genes to generate high affinity variants.

Bass et al., 1990, Proteins: Struct. Func. Genet. 8:309-314 fused human growth hormone (hGH) to the carboxy
30 terminus of the gene III protein of phage fd. This fusion

protein was built into a phagemid vector. When cells carrying the phagemid were infected with a helper phage, about 10% of the phage particles produced displayed the fusion protein on their surfaces. These phage particles were enriched by screening with hGH receptor-coated beads. It was suggested that this system could be used to develop mutants of hGH with altered receptor binding characteristics.

Lowman et al., 1991, Biochemistry 30:10832-10838 used an improved version of the system of Bass et al. described above to select for mutant hGH proteins with exceptionally high affinity for the hGH receptor. The authors randomly mutagenized the hGH-pIII fusion proteins at sites near the vicinity of 12 amino acids of hGH that had previously been identified as being important in receptor binding.

Balass et al., 1993, Proc. Natl. Acad. Sci. USA 90:10638-10642 used a phage display library to isolate linear peptides that mimicked a conformationally dependent epitope of the nicotinic acetylcholine receptor. This was done by screening the library with a monoclonal antibody specific for the conformationally dependent epitope. The monoclonal antibody used was thought to be specific to the acetylcholine receptor's binding site for its natural ligand, acetylcholine.

2.2. Drug Delivery Systems

The common routes of therapeutic drug administration are oral ingestion or parenteral (intravenous, subcutaneous and intramuscular) routes of administration. Intravenous drug administration suffers from numerous limitations, including (i) the risk of adverse effects resulting from rapid accumulation of high concentrations of drug, (ii) repeated injections which can cause patient discomfort; and (iii) the risk of infection at the site of repeated injections. Subcutaneous injection is not generally suitable for delivering large volumes or for irritating

substances. Whereas oral administration is generally more convenient, it is limited where the therapeutic agent is not efficiently absorbed by the gastrointestinal tract. To date, the development of oral formulations for the effective delivery of peptides, proteins and macromolecules has been an elusive target. Poor membrane permeability, enzymatic instability, large molecular size, and hydrophilic properties are four factors that have remained major hurdles for peptide and protein formulations (reviewed by Fix, J.A., 1996, J. Pharmac. Sci. 85:1282-1285). In order to develop an efficacious oral formulation, the peptide must be protected from the enzymatic environment of the gastrointestinal tract (GIT), presented to the absorptive epithelial barrier in a sufficient concentration to effect transcellular flux (Fix, J.A., 1996, J. Pharmac. Sci. 85:1282-1285), and if possible "smuggled" across the epithelial barrier in an apical to basolateral direction.

Site specific drug delivery or drug targeting can be achieved at different levels, including (i) primary targeting to a specific organ, (ii) secondary targeting to a specific cell type within that organ and (iii) tertiary targeting where the drug is delivered to specific intracellular structures (e.g., the nucleus for genes) (reviewed in Davis and Jllum, 1994, In: Targeting of Drugs 4, (Eds), Gregoriadis, McCormack and Poste, 183-194). At present there is a considerable amount of ongoing research work in the Drug Delivery Systems (DDS) area, and much of it addresses (i) targeting delivery and (ii) the development of non-invasive ways of getting macromolecules, peptides, proteins, products of the biotechnology industry, etc. into the body (Evers, P., 1995, Developments in Drug Delivery: Technology and Markets, Financial Times Management Report). It is generally accepted that targeted drug delivery is crucial to the improved treatment of certain diseases, especially cancer, and not surprisingly many of the approaches to targeted drug delivery are focused in the

cancer area. Many anticancer drugs are toxic to the body as well as to malignant cells. If a drug, or a delivery system, can be modified so that it "homes in" on the tumor, then by maximizing the drug concentration at the disease site, the anti-cancer effect can be exploited to the full, while
5 toxicity is greatly reduced. Tumors contain antigens which provoke the body to respond by producing antibodies designed to attach to the antigens and destroy them. Monoclonal antibodies are being used as both delivery vehicles targeted to tumor cells (reviewed by Pietersz, G.A., 1990, Bioconjugate Chem. 1:89-95) and as imaging agents to carry
10 molecules of drug or imaging agent to the tumor surface.

2.3. Transport Pathways

The epithelial cells lining the luminal side of the GIT are a major barrier to drug delivery following oral administration. However, there are four recognized transport
15 pathways which can be exploited to facilitate drug delivery and transport: the transcellular, paracellular, carrier-mediated, and transcytotic pathways. The ability of a conventional drug, peptide, protein, macromolecule or nano-or microparticulate system to "interact" with one of these transport pathways may result in increased delivery of
20 that drug or particle from the GIT to the underlying circulation.

In the case of the receptor-mediated, carrier-mediated or transcytotic transport pathways, some of the uptake signals have been identified. These signals include, *inter alia*, folic acid, which interacts with the
25 folate receptor, and cobalamin, which interacts with Intrinsic Factor. In addition, leucine- and tyrosine-based peptide sorting motifs or internalization sequences exist, such as YSKV, FPHL, YRGV, YQTI, TEQF, TEVM, TSAF, and YTRF (SEQ ID NOS:203, 204, 205, 206, 207, 208, 209, and 210,
30 respectively), which facilitate uptake or targeting of proteins using specific membrane receptors or binding sites

to identify peptides that bind specifically to the receptor or binding site.

Non-receptor based assays to discover particular ligands have also been used. For instance, a strategy for identifying peptides that alter cellular function by scanning
5 whole cells with phage display libraries is disclosed in Fong et al., Drug Development Research 33:64-70 (1994). However, because whole cells, rather than intact tissue or polarized cell cultures, are used for screening phage display libraries, this procedure does not provide information regarding sequences whose primary function includes affecting
10 transport across polarized cell layers.

Additionally, Stevenson et al., Pharmaceutical Res. 12(9), S94 (1995) discloses the use of Caco-2 monolayers to screen a synthetic tripeptide combinatorial library for information relating to the permeability of di- and
15 tri-peptides.

A method of identifying a peptide which permits or facilitates the transport of an active agent through human or animal tissues has been developed (see U.S. patent application Serial No. 08/746,411 filed November 8, 1996, which is incorporated by reference herein in its entirety).
20 Phage from a random phage library is plated onto or brought into contact with a first side, preferably the apical side, of a tissue sample, either *in vitro*, *in vivo* or *in situ*, or polarized tissue cell culture. The phage which is transported to a second side of the tissue opposite the first side, preferably the basolateral side, is harvested to select
25 transported phages. The transported phages are amplified in a host and this cycle is repeated (using the transported phage from the most recent cycle) to obtain a selected phage library containing phage which can be transported from the first side to the second side.

Discussion or citation of a reference hereinabove
30 shall not be construed as meaning that such reference is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates generally to random peptides and peptide motifs capable of specific binding to GIT transport receptors. Such proteins can be identified using any random peptide library, e.g., a chemically
5 synthesized peptide library or a biologically expressed peptide library. If a biological peptide expression library is used, the nucleic acid which encodes the peptide which binds to the ligand of choice can be recovered, and then sequenced to determine its nucleotide sequence and hence
10 deduce the amino acid sequence that mediates binding. Alternatively, the amino acid sequence of an appropriate binding domain can be determined by direct determination of the amino acid sequence of a peptide selected from a peptide library containing chemically synthesized peptides. In a less preferred aspect, direct amino acid sequencing of a
15 binding peptide selected from a biological peptide expression library can also be performed.

In particular, this invention relates to proteins (e.g., peptides) that are capable of facilitating transport of an active agent through a human or animal gastro-intestinal tissue, and derivatives (e.g., fragments)
20 and analogs thereof, and nucleotide sequences coding for said proteins and derivatives.

Preferably, the tissue through which transport is facilitated is of the duodenum, jejunum, ileum, ascending colon, transverse colon, descending colon, or pelvic colon. The tissue is most preferably epithelial cells lining the
25 lumenal side of the GIT.

The proteins of the invention have use in facilitating transport of active agents from the lumenal side of the GIT into the systemic blood system, and/or in targeting active agents to the GIT. Thus, for example, by binding (covalently or noncovalently) a protein of the
30 invention to an orally administered drug, the drug can be targeted to specific receptor sites or transport pathways

which are known to operate in the human gastrointestinal tract, thus facilitating its absorption into the systemic system.

The invention also relates to derivatives and analogs of the invention which are functionally active, i.e.,
5 they are capable of displaying one or more known functional activities associated with a full-length peptide. Such functional activities include but are not limited to antigenicity (ability to bind or to compete with GIT transport receptor-binding peptides for binding to an anti-GIT transport receptor antibody) and ability to bind or
10 compete with full-length peptide for binding to a GIT transport receptor.

The invention further relates to fragments of (and derivatives and analogs thereof) GIT transport receptor-binding peptides which comprise one or more motifs of a GIT transport receptor-binding peptide.
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Antibodies to GIT transport receptor-binding peptides and GIT transport receptor-binding peptide derivatives and analogs are additionally provided.

Methods of production of the GIT transport receptor-binding peptides, derivatives, fragments and analogs, e.g., by recombinant means, are also provided.
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The present invention also relates to therapeutic methods, pharmaceutical compositions and formulations based on GIT transport receptor-binding peptides. Formulations of the invention include but are not limited to GIT transport receptor-binding peptides or motifs and derivatives
25 (including fragments) thereof; antibodies thereto; and nucleic acids encoding the GIT transport receptor-binding peptides or derivatives associated with an active agent. Preferably, the active agent is a drug or drug-containing nano- or microparticle.

The GIT transport-receptor binding proteins of the invention can also be used to determine levels of the GIT
30 transport receptors in a sample by binding thereto.

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The GIT transport-receptor binding proteins can also be used to identify molecules that bind thereto, by contacting candidate test molecules under conditions conducive to binding, and detecting any binding that occurs.

5 **4. DESCRIPTION OF THE FIGURES**

Figure 1. Figure 1 shows the human PEPT1 predicted amino acid sequence determined from the sequence of the cDNA clone coding for human PEPT1 (SEQ ID NO:176) (Liang R. et al. J. Biol. Chem. 270(12):6456-6463 (1995)), including the
10 extracellular domain from amino acid 391 to 573 (Fei et al., Nature 368:563 (1994)).

Figures 2A-2C. Figures 2A-2C show the DNA sequence of the cDNA coding for the human intestinal peptide-associated transporter HPT1 and the corresponding putative amino acid sequence (bases 1 to 3345; Medline:94204643) (SEQ ID NOS: 177
15 and 178, respectively).

Figures 3A-3B. Figures 3A-3B show the putative Human Sucrase-isomaltase complex(hSI) amino acid sequence determined from the sequence of the cDNA clone coding for human sucrase-isomaltase complex (SEQ ID NO:179) (Chantret I., et al., Biochem. J. 285(Pt 3):915-923 (1992)).

20 **Figures 4A-4B.** Figures 4A-4B show the D2H nucleotide and deduced amino acid sequence for the human D2H transporter (SEQ ID NOS:180 and 181, respectively) (Wells, R.G. et al., J. Clin. Invest. 90:1959-1963 (1993)).

Figures 5A-5C. Figure 5A is a schematic summary of the
25 cloning of the DNA insert present in gene III of the phages selected from the phage display libraries into the expression vector pGex-4T-2. The gene insert in gene III of the phages was amplified by PCR using DNA primers which flank the gene insert and which contained recognition sequences for specific restriction endonucleases at their extreme 5' sides.

30 Alternatively, specific primers which amplify specific regions of the DNA inserts in gene III of the phages, and

which contained recognition sequences for specific
 restriction endonucleases at their extreme 5' sides, were
 used in PCR amplification experiments. Following
 amplification of the gene inserts, the amplified PCR
 fragments were digested with the restriction endonucleases
 5 Xho1 and Not1. Similarly the plasmid pGex-4T-2, which codes
 for the reporter protein glutathione S-transferase (GST), was
 digested with the restriction endonucleases Sal1 and Not1.
 The digested PCR fragments were ligated into the digested
 plasmid pGex-4T-2 using T4 DNA Ligase and the ligated
 products were transformed into competent *Escherichia coli*,
 10 with selection of transformants on agar plates containing
 selection antibiotic. The selected clones were cultured, the
 plasmids were recovered and the in-frame sequence of the DNA
 insert in the plasmids was confirmed by DNA sequencing. The
 correct clones were subsequently used for expression of the
 15 GST-fusion proteins (SEQ ID NO:182); Figure 5B shows the
 series of full-length P31 (designated P31) (SEQ ID NO:43) and
 truncated peptides derived from P31 (clones # 101, 102, 103
 and 119), (SEQ ID NOS:183, 184, 185, and 186, respectively)
 full-length PAX2 (designated PAX2) (SEQ ID NO:55) and
 truncated peptides derived from PAX2 (clones # 104, 105, 106)
 (SEQ ID NOS:170, 187, and 188, respectively) and full-length
 20 DCX8 (DCX8) (SEQ ID NO:23) and series of truncated peptides
 derived from DCX8 (clones # 107, 108, 109) (SEQ ID NOS:189,
 190, and 191, respectively) that were expressed as fusion
 proteins to GST. The construction of these GST-fusion
 proteins is shown in Figure 5A. Figure 5C shows the series
 of full-length P31 (designated P31) (SEQ ID NO:43) and
 25 truncated peptides derived from P31 (clones # 103, 110, 119,
 111, and 112) (SEQ ID NOS:185, 192, 186, 194, and 195,
 respectively), full-length PAX2 (designated PAX2) (SEQ ID
 NO:55) and truncated peptides derived from PAX2 (clones #
 106, 113, 114, 115) (SEQ ID NOS:188, 196, 197, and 198,
 respectively) and full-length SNI10 (designated SNI10) (SEQ
 30 ID NO:4) and series of truncated peptides derived from SNI10

(clones # 116, 117, 118) (SEQ ID NOS:199, 200, and 405, respectively) that were expressed as fusion proteins to GST. The construction of these GST-fusion proteins is shown in Figure 5A. (Underlining and bold in Figs. 5A-5C are for orientation of the sequences.)

5 **Figures 6A-6B.** Figures 6A-6B show the binding of GST and GST-fusion proteins to recombinant hSI and to fixed C2BBel fixed cells as detected by ELISA assays. Figure 6A shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from SNI10 (designated GST-SNI10) and SNI34 (designated GST-SNI34) to
10 recombinant hSI. Figure 6B shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from SNI10 (designated GST-SNI10) and SNI34 (designated GST-SNI34) to fixed C2BBel cells.

Figures 7A-7M. Figures 7A-7M show the binding of GST peptide
15 and truncated fusion proteins to fixed Caco-2 cells, fixed C2BBel cells, and fixed A431 cells or to recombinant GIT transport receptors D2H, HPT1, hPEPT1 or to BSA using increasing concentrations (expressed as $\mu\text{g/ml}$ on the X-axis) of the control GST protein and the GST-fusion proteins, as detected by ELISA assays. Figure 7A shows the binding of the
20 control protein GST, which does not contain a fusion peptide, and the series of GST-fusion proteins from P31 including the fusion to full-length P31 peptide (designated P31) (SEQ ID NO:43) and clone # 101 (designated P31,101, SEQ ID NO: 183), clone # 102 (designated P31, 102 SEQ ID NO: 184) and clone # 103 (designated P31,103 SEQ ID NO: 185). Figure 7B shows
25 the binding of the control protein GST, which does not contain a fusion peptide, and the series of GST-fusion proteins from PAX2 including the fusion to full-length PAX2 peptide (designated PAX2) and clone # 104 (designated PAX2,104), clone # 105 (designated PAX2, 105) and clone # 106 (designated PAX2,106) (SEQ ID NOS:55, 170, 187, and 188,
30 respectively). Figure 7C shows the binding of the control protein GST, which does not contain a fusion peptide, and the

series of GST-fusion proteins from DCX8 including the fusion to full-length DCX8 peptide (designated DCX8) and clone # 107 (designated DCX8,107), clone # 108 (designated DCX8, 108) and clone # 109 (designated DCX8,109) (SEQ ID NOS:23, 189, 190, and 191, respectively). Figure 7D shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from DCX8 (designated GST-DCX8) and DCX11 (designated GST-DCX11) to recombinant D2H. Figure 7E shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from DCX8 (designated GST-DCX8) and DCX11 (designated GST-DCX11) to fixed C2BBel cells. Figure 7F shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from P31 (designated GST-P31) and 5PAX5 (designated GST-5PAX5) to recombinant hPEPT1. Figure 7G shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from P31 (designated GST-P31) and 5PAX5 (designated GST-5PAX5) to fixed C2BBel cells. Figure 7H shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from HAX42 (designated GST-HAX42) and PAX2 (designated GST-PAX2) to recombinant HPT1. Figure 7I shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from HAX42 (designated GST-HAX42) and PAX2 (designated GST-PAX2) to fixed C2BBel cells. Figure 7J shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from P31 (designated GST-P31) and truncated derivatives clone # 101 (designated GST-P31-101 , SEQ ID NO: 183), clone # 102 (designated GST-P31-102 , SEQ ID NO: 184), clone # 103 (designated GST-P31-103 , SEQ ID NO: 185) to either recombinant hPEPT1 or to BSA. Figure 7K shows the binding of the control protein GST, which does not contain a fusion peptide, and the GST-fusion proteins from P31 (designated GST-P31) and truncated derivatives clone # 101 (designated GST-P31-101 , SEQ ID NO: 183), clone # 102

(designated GST-P31-102 , SEQ ID NO: 184), clone # 103
(designated GST-P31-103 , SEQ ID NO: 185) to either fixed
C2BBel cells or to fixed A431 cells. Figure 7L shows the
binding of the control protein GST, which does not contain a
fusion peptide, and the GST-fusion proteins from PAX2
5 (designated GST-PAX2) and truncated derivatives clone # 104
(designated GST-PAX2-104 , SEQ ID NO: 170), clone # 105
(designated GST-PAX2-105 , SEQ ID NO: 187), clone # 106
(designated GST-PAX2-106 , SEQ ID NO: 188) to either
recombinant hPEPT1 or to BSA. Figure 7M shows the binding of
the control protein GST, which does not contain a fusion
10 peptide, and the GST-fusion proteins from PAX2 (designated
GST-PAX2) and truncated derivatives clone # 106 (designated
GST-PAX2-106 , SEQ ID NO: 188) to either fixed Caco-2 cells
or to fixed A431 cells. .
Figures 8A-8D. Figure 8 shows the transport of GST or
GST-peptide fusion derivatives across polarized Caco-2 cells
15 in an apical to basolateral direction as a function of time
(1-4 hours) as detected by ELISA assays. Figure 8A shows the
transport of either GST, the GST fusion to full-length P31
peptide (designated P31) (SEQ ID NO:43) and the GST clone
derivative clone # 103 (designated P31.103 , SEQ ID NO: 185)
across polarized Caco-2 cells in an apical to basolateral as
20 a function of time (in hours) following initial
administration of the proteins to the apical medium of
polarized Caco-2 cells. The line designated No Protein
corresponds to control assays in which buffer control was
applied to the apical medium of polarized Caco-2 cells
followed by sampling of the basolateral medium as a function
25 of time (hours) and assay for GST by the ELISA assay. Figure
8B shows the transport of either GST, the GST fusion to
full-length PAX2 peptide (designated PAX2) and the GST clone
derivative clone # 106 (designated PAX2.106 , SEQ ID NO: 188)
across polarized Caco-2 cells in an apical to basolateral as
a function of time (in hours) following initial
30 administration of the proteins to the apical medium of

polarized Caco-2 cells. The line designated No Protein corresponds to control assays in which buffer control was applied to the apical medium of polarized Caco-2 cells followed by sampling of the basolateral medium as a function of time (hours) and assay for GST by the ELISA assay. Figure 8C shows the transport of either GST, the GST fusion to full-length DCX8 peptide (designated DCX8), and the GST clone derivatives clone # 107 (designated DCX8.107) and clone # 109 (designated DCX8.109) across polarized Caco-2 cells in an apical to basolateral as a function of time (in hours) following initial administration of the proteins to the apical medium of polarized Caco-2 cells. The line designated No Protein corresponds to control assays in which buffer control was applied to the apical medium of polarized Caco-2 cells followed by sampling of the basolateral medium as a function of time (hours) and assay for GST by the ELISA assay. Figure 8D shows the amount of the GST and GST-fusion proteins (GST fusions to P31, P31-103 (SEQ ID NO: 183), PAX2, PAX2.106 (SEQ ID NO: 188), DCX8, DCX8-107, DCX8-109), used in the experiments shown in panels A-C above, in the apical medium of the polarized Caco-2 cells as detected by ELISA assay.

Figures 9A-9B. Figures 9A-9B show the inhibition of GST-P31 binding to C2BBel fixed cells with varying concentration of competitors while holding the concentration of GST-P31 constant at 0.015 μ M; the peptide competitors are ZElan024 (SEQ ID NO:288) which is the dansylated peptide version of P31 (SEQ ID NO:43) and ZElan044 (SEQ ID NO:310), ZElan049 (SEQ ID NO:315) and ZElan050 (SEQ ID NO:316) which are truncated, dansylated pieces of P31 (SEQ ID NO:43). Data is presented as O.D. versus peptide concentration (Figure 9A) and as percent inhibition of GST-P31 binding versus peptide concentration (Figure 9B).

Figures 10A-10C. Figures 10A-10C present a compilation of the results of competition ELISA studies of GST-P31, GST-PAX2, GST-SNi10 and GST-HAX42 versus listed dansylated

peptides on fixed C2BBel cells ("Z" denotes ϵ -amino dansyl lysine). The pI of the dansylated peptides is also included. Estimated IC₅₀ values are in μ M and where present, IC₅₀ ranges refer to results from multiple assays. If the IC₅₀ value could not be determined, a ">" or "<" symbol is used. The
5 GST/C2BBel column shows GST protein binding to fixed C2BBel cells.

Figures 11A-11B. Figure 11A shows the transport of GST or GST-peptide fusion derivatives across polarized Caco-2 cells in an apical to basolateral direction at 0, 0.5, 2 and 4 hours as detected by ELISA assays and described elsewhere in
10 the text in full detail. The proteins used in the assay included GST, GST-P31 fusion, GST-5PAX5 fusion, GST-DCX8 fusion, GST-DCX11 fusion, GST-PAX2 fusion, GST-HAX42 fusion, GST-SNi34 fusion and GST-SNi10 fusion. The column designated No protein refers to control experiments in which buffer was applied to the apical medium of the cells and ELISA assay was
15 performed on the corresponding basolateral medium of these cells at 0, 0.5, 2 and 4 hours post buffer addition. Figure 11B shows the internalization of GST or GST-peptide fusion derivatives within polarized Caco-2 cells following administration of the GST or GST-fusion protein derivatives to the apical medium of polarized Caco-2 cells and subsequent
20 recovery of the cells from the transwells and detection of the GST or GST fusions within the recovered cell lysates as detected by ELISA assays and as described elsewhere in the text in full detail. The proteins used in the assay included GST, GST-P31 fusion, GST-5PAX5 fusion, GST-DCX8 fusion, GST-DCX11 fusion, GST-PAX2 fusion, GST-HAX42 fusion,
25 GST-SNi34 fusion and GST-SNi10 fusion. The column designated No protein refers to control experiments in which buffer was applied to the apical medium of the cells and ELISA assay was performed on the corresponding cell lysates of these cells at the end of the experiment.

Figure 12. Figure 12 shows the binding of GST and GST-fusion
30 proteins to fixed Caco-2 cells, and the corresponding

proteins following digestion with the protease Thrombin which cleaves at a recognition site between the GST portion and the fused peptide portion of the GST-fusion protein. The symbol "-" refers to proteins which were not digested with thrombin and the symbol "+" refers to proteins which were digested
5 with thrombin prior to use in the binding assay. The binding of the proteins to the fixed Caco-2 cells was detected by ELISA assays.

Figures 13A-13B. Figures 13A-13B show binding of peptide-coated nanoparticles to fixed Caco-2 cells.

Figures 14A-14B. Figures 14A-14B show the binding of (A)
10 dansylated peptide SNI10 to the purified hSI receptor and BSA and (B) dansylated peptides and peptide-loaded insulin-containing PLGA particles to fixed C2BBel cells. Figure 14B depicts binding of dansylated peptides corresponding to P31 (SEQ ID NO:43), PAX2, HAX42, and SNI10
15 to fixed C2BBel cells, as well as the insulin-containing PLGA particles adsorbed with each of these peptides. Data is presented with background subtracted.

Figures 15A-15B. Figure 15 shows the binding of peptide-coated particles to A) S100 and B) P100 fractions harvested from Caco-2 cells. The dilution series 1:2 - 1:64
20 represents particle concentrations in the range 0.0325-0.5 μ g/well. Data is presented with background subtracted. The particles are identified as follows: 939, no peptide; 1635, scrambled PAX2; 1726, P31 D-Arg 16-mer (ZElan053 ,SEQ ID NO:319); 1756, HAX42; 1757, PAX2; 1758, HAX42/PAX2.

Figures 16A-16B. Figure 16 shows the binding of dansylated
25 peptides to P100 fractions harvested from Caco-2 cells. Peptides were assayed in the range 0.0032-2.5 μ g/well. Data is presented with background subtracted. A) HAX42, P31 D-form (ZElan 053 , (SEQ ID NO:319) and scrambled PAX2; B) PAX2, HAX42 and scrambled PAX2.

Figures 17A-17B. Figures 17A and 17B show (A) the systemic
30 blood glucose and (B) insulin levels following intestinal administration of control (PBS); insulin solution; insulin

particles; all 8 peptides mix particles and study group peptide-particles according to this invention (100iu insulin loading).

Figures 18A-18B. Figures 18A and 18B show the (A) systemic blood glucose and (B) insulin levels following intestinal administration of control (PBS); insulin solution; insulin particles and study group peptide-particles according to this invention (300iu insulin loading).

Figure 19. Figure 19 shows the enhanced plasma levels of leuprolide upon administration of P31 (SEQ ID NO:43) and PAX2 coated nanoparticles loaded with leuprolide relative to subcutaneous injection. Group 1 was administered leuprolide acetate (12.5 μ g) subcutaneously. Group 2 was administered intraduodenally uncoated leuprolide acetate particles (600 μ g, 1.5 ml). Group 3 was intraduodenally administered leuprolide acetate particles coated with PAX2 (600 μ g; 1.5 ml). Group 4 was administered intraduodenally leuprolide acetate particles coated with P31 (SEQ ID NO:43) (600 μ g, 1.5 ml).

Figure 20. Figure 20 lists P31 (SEQ ID NO:43) known protein homologies.

Figures 21A-21C. Figures 21A-21C list DCX8 known protein homologies.

Figure 22. Figure 22 lists DAB10 known protein homologies.

Figure 23. Figure 23 shows the DNA sequence (SEQ ID NO:211) and the corresponding amino acid sequence (SEQ ID NO:212) for glutathione S-transferase (Smith and Johnson, 1988, Gene 7:31-40).

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to proteins (e.g., peptides) that bind to GIT transport receptors and nucleic acids that encode such proteins. The invention further relates to fragments and other derivatives of such proteins. Nucleic acids encoding such fragments or derivatives are also

within the scope of the invention. The invention further relates to fragments (and derivatives and analogs thereof) of GIT transport receptor-binding peptides which comprise one or more domains of the GIT transport receptor-binding peptides.

The invention also relates to derivatives of GIT
5 transport receptor-binding proteins and analogs of the invention which are functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length GIT transport receptor-binding peptide. Such functional activities include but are not limited to ability to bind to a GIT transport receptor,
10 antigenicity [ability to bind (or compete with peptides for binding) to an anti-GIT transport receptor-binding peptide antibody], immunogenicity (ability to generate antibody which binds to GIT transport receptor-binding peptide), etc.

Production of the foregoing proteins and
15 derivatives, by, e.g., recombinant methods, is also provided.

Antibodies to GIT transport receptor-binding proteins, derivatives and analogs, are additionally provided.

The present invention also relates to therapeutic and diagnostic methods and compositions based on GIT transport receptor-binding proteins and nucleic acids.

The invention is illustrated by way of examples
20 *infra*.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

25 **5.1. GIT Transport Receptor-Binding Peptides, Derivatives and Analogs**

The invention relates to peptides that bind GIT transport receptors and derivatives (including but not limited to fragments) and analogs thereof. In specific embodiments, of the present invention, such peptides that
30 bind to GIT transport receptor include but are not limited to those containing as primary amino acid sequences, all or part

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of the amino acid sequences substantially as depicted in Table 7 (SEQ ID NOS:1-55). Nucleic acids encoding such peptides, derivatives and peptide analogs are also provided. In one embodiment, the GIT transport receptor-binding peptides are encoded by the nucleic acids having the

5 nucleotide sequences set forth in Table 8 *infra* (SEQ ID NOS:56-109). Proteins whose amino acid sequence comprise, or alternatively, consist of SEQ ID NOS:1-55 or a portion thereof that mediates binding to a GIT transport receptor are provided.

10 The production and use of derivatives and analogs related to GIT transport receptor-binding peptides are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with a full-length GIT transport receptor-binding

15 peptide. For example, such derivatives or analogs which have the desired immunogenicity or antigenicity can be used, in immunoassays, for immunization, etc. A specific embodiment relates to a GIT transport receptor-binding peptide fragment that can be bound by an anti-GIT transport receptor-binding peptide antibody. In a preferred aspect, the derivatives or

20 analogs have the ability to bind to a GIT transport receptor. Derivatives or analogs of GIT transport receptor-binding peptides can be tested for the desired activity by procedures known in the art, including binding to a GIT transport receptor domain or to Caco-2 cells, *in vitro*, or to intestinal tissue, *in vivo*. (See the Examples *infra*.)

25 In particular, derivatives can be made by altering GIT transport receptor-binding peptide sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other nucleotide sequences which encode substantially the same amino acid sequence may be used

30 in the practice of the present invention. These include but are not limited to nucleotide sequences which are altered by

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the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the GIT transport receptor-binding peptide derivatives of the invention include, but are not limited to, those containing, 5 as a primary amino acid sequence, all or part of the amino acid sequence of a GIT transport receptor-binding peptide including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be 10 substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, 15 isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and 20 glutamic acid.

In a specific embodiment of the invention, proteins consisting of or, alternatively, comprising all or a fragment of a GIT transport receptor-binding peptide consisting of at least 5, 10, 15, 20, 25, 30 or 35 (contiguous) amino acids of the full-length GIT transport receptor-binding peptide are 25 provided. In a specific embodiment, such proteins are not more than 20, 30, 40, 50, or 75 amino acids in length. Derivatives or analogs of GIT transport receptor-binding peptides include but are not limited to those molecules comprising regions that are substantially homologous to GIT transport receptor-binding peptides or fragments thereof 30 (e.g., at least 50%, 60%, 70%, 80% or 90% identity) (e.g.,

over an identical size sequence or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a coding GIT transport receptor-binding peptide sequence, under stringent,
5 moderately stringent, or nonstringent conditions.

In a specific embodiment, the GIT transport receptor-binding derivatives of the invention are not known proteins with homology to the GIT transport receptor-binding peptides of the invention or portions thereof.

The GIT transport receptor-binding peptide
10 derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned GIT transport receptor-binding peptide gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990,
15 Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the
20 production of the gene encoding a derivative or analog of GIT transport receptor-binding peptides, care should be taken to ensure that the modified gene remains within the same translational reading frame uninterrupted by translational stop signals, in the gene region where the desired GIT transport receptor-binding peptides activity is encoded.

Additionally, nucleic acid sequences encoding the
25 GIT transport receptor-binding peptides can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate
30 further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to,

chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), use of PCR primers containing mutation(s) for use in amplification, etc.

5 Manipulations of GIT transport receptor-binding peptide sequences may also be made at the protein level. Included within the scope of the invention are GIT transport receptor-binding peptide fragments or other derivatives or analogs which are differentially modified during or after translation or chemical synthesis, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by
10 known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation,
15 formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc. In a specific embodiment, the amino- and/or carboxy-termini are modified.

In addition, GIT transport receptor-binding peptides and analogs and derivatives thereof can be chemically synthesized. For example, a peptide corresponding
20 to all or a portion of a GIT transport receptor-binding peptide which comprises the desired domain or which mediates the desired activity *in vitro*, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced
25 as a substitution or addition into the GIT transport receptor-binding peptide sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ -Abu, ϵ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid,
30 ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine,

phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β -methyl amino acids, $\text{C}\alpha$ -methyl amino acids, $\text{N}\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

5 In a specific embodiment, the GIT transport
receptor-binding peptide derivative is a chimeric, or fusion,
peptide comprising a GIT transport receptor-binding peptide
or fragment thereof (preferably consisting of at least a
domain or motif of the GIT transport receptor-binding
10 peptide, or at least 6, 10, 15, 20, 25, 30 or all amino acids
of the GIT transport receptor-binding peptides or a binding
portion thereof) joined at its amino- or carboxy-terminus via
a peptide bond to an amino acid sequence of a different
peptide. In one embodiment, such a chimeric peptide is
produced by recombinant expression of a nucleic acid encoding
the protein (comprising a transport receptor-coding sequence
15 joined in-frame to a coding sequence for a different
protein). Such a chimeric product can be made by ligating
the appropriate nucleic acid sequences encoding the desired
amino acid sequences to each other by methods known in the
art, in the proper coding frame, and expressing the chimeric
product by methods commonly known in the art. Alternatively,
20 such a chimeric product may be made by protein synthetic
techniques, e.g., by use of a peptide synthesizer. Chimeric
genes comprising portions of GIT transport receptor fused to
any heterologous protein-encoding sequences may be
constructed. A specific embodiment relates to a chimeric
protein comprising a fragment of GIT transport
25 receptor-binding peptides of at least six amino acids.

In another specific embodiment, the GIT transport receptor-binding peptide derivative is a molecule comprising a region of homology with a GIT transport receptor-binding peptide. By way of example, in various embodiments, a first protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first

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region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, or 95% identical, when compared to any sequence in the second region of an equal number of amino acids as the number contained in the first region or when compared to an aligned sequence of the second region that has been aligned by a
5 computer homology program known in the art. For example, a molecule can comprise one or more regions homologous to a GIT transport receptor-binding peptide domain (see *infra*) or a portion thereof.

The GIT transport receptor-binding proteins and derivatives thereof of the invention can be assayed for
10 binding activity by suitable *in vivo* or *in vitro* assays, e.g., as described in the examples *infra* and/or as will be known to the skilled artisan.

Other specific embodiments of derivatives and analogs are described in the subsection below and examples
15 sections *infra*.

5.2. Motifs/Derivatives of GIT Transport Receptor-Binding Peptides Containing One or More Domains of The Protein

In a specific embodiment, the invention relates to
20 GIT transport receptor-binding peptide derivatives and analogs, in particular GIT transport receptor-binding peptide fragments and derivatives of such fragments, that comprise, or alternatively consist of, one or more domains of a GIT transport receptor-binding peptide. In particular, examples of such domains are identified in the examples *infra*.

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5.3. Synthesis of Peptides

The peptides and derivatives of the present invention may be chemically synthesized or synthesized using recombinant DNA techniques.

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5.3.1. Procedure For Solid Phase Synthesis

Peptides may be prepared chemically by methods that are known in the art. For example, in brief, solid phase peptide synthesis consists of coupling the carboxyl group of the C-terminal amino acid to a resin and successively adding N-alpha protected amino acids. The protecting groups may be
5 any known in the art. Before each new amino acid is added to the growing chain, the protecting group of the previous amino acid added to the chain is removed. The coupling of amino acids to appropriate resins is described by Rivier et al., U.S. Patent No. 4,244,946. Such solid phase syntheses have been described, for example, by Merrifield, 1964, J. Am.
10 Chem. Soc. 85:2149; Vale et al., 1981, Science 213:1394-1397; Marki et al., 1981, J. Am. Chem. Soc. 103:3178 and in U.S. Patent Nos. 4,305,872 and 4,316,891. In a preferred aspect, an automated peptide synthesizer is employed.

By way of example but not limitation, peptides can be synthesized on an Applied Biosystems Inc. ("ABI") model
15 431A automated peptide synthesizer using the "Fastmoc" synthesis protocol supplied by ABI, which uses 2-(1H-Benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate ("HBTU") (R. Knorr et al., 1989, Tet. Lett., 30:1927) as coupling agent. Syntheses can be carried out on 0.25 mmol of commercially available
20 4-(2',4'-dimethoxyphenyl-(9-fluorenyl-methoxycarbonyl)-aminomethyl)-phenoxy polystyrene resin ("Rink resin" from Advanced ChemTech) (H. Rink, 1987, Tet. Lett. 28:3787). Fmoc amino acids (1 mmol) are coupled according to the Fastmoc protocol. The following side chain protected Fmoc amino acid derivatives are used: FmocArg(Pmc)OH; FmocAsn(Mbh)OH;
25 FmocAsp(^tBu)OH; FmocCys(Acm)OH; FmocGlu(^tBu)OH; FmocGln(Mbh)OH; FmocHis(Tr)OH; FmocLys(Boc)OH; FmocSer(^tBu)OH; FmocThr(^tBu)OH; FmocTyr(^tBu)OH. [Abbreviations: Acm, acetamidomethyl; Boc, tert-butoxycarbonyl; ^tBu, tert-butyl; Fmoc, 9-fluorenylmethoxycarbonyl; Mbh, 4,4'-dimethoxybenzhydryl; Pmc,
30 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Tr, trityl].

Synthesis is carried out using N-methylpyrrolidone (NMP) as solvent, with HBTU dissolved in N,N-dimethylformamide (DMF). Deprotection of the Fmoc group is effected using approximately 20% piperidine in NMP. At the end of each synthesis the amount of peptide present is
5 assayed by ultraviolet spectroscopy. A sample of dry peptide resin (about 3-10 mg) is weighed, then 20% piperidine in DMA (10 ml) is added. After 30 min sonication, the UV (ultraviolet) absorbance of the dibenzofulvene-piperidine adduct (formed by cleavage of the N-terminal Fmoc group) is recorded at 301 nm. Peptide substitution (in mmol g⁻¹) can be
10 calculated according to the equation:

$$\text{substitution} = \frac{A \times v}{7800 \times w} \times 1000$$

where A is the absorbance at 301 nm, v is the volume of 20% piperidine in DMA (in ml), 7800 is the extinction coefficient
15 (in mol⁻¹dm³cm⁻¹) of the dibenzofulvene-piperidine adduct, and w is the weight of the peptide-resin sample (in mg).

Finally, the N-terminal Fmoc group is cleaved using 20% piperidine in DMA, then acetylated using acetic anhydride and pyridine in DMA. The peptide resin is thoroughly washed with DMA, CH₂Cl₂ and finally diethyl ether.

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5.3.2. Cleavage And Deprotection

By way of example but not limitation, cleavage and deprotection can be carried out as follows: The air-dried peptide resin is treated with ethylmethyl-sulfide (EtSMe), ethanedithiol (EDT), and thioanisole (PhSMe) for
25 approximately 20 min. prior to addition of 95% aqueous trifluoroacetic acid (TFA). A total volume of approximately 50 ml of these reagents per gram of peptide-resin is used. The following ratio is used: TFA:EtSMe:EDT:PhSMe (10:0.5:0.5:0.5). The mixture is stirred for 3 h at room temperature under an atmosphere of N₂. The mixture is
30 filtered and the resin washed with TFA (2 x 3 ml). The combined filtrate is evaporated *in vacuo*, and anhydrous

diethyl ether added to the yellow/orange residue. The resulting white precipitate is isolated by filtration. See King et al., 1990, Int. J. Peptide Protein Res. 36:255-266 regarding various cleavage methods.

5 **5.3.3. Purification of the Peptides**

Purification of the synthesized peptides can be carried out by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography, high performance liquid chromatography (HPLC)), centrifugation, differential solubility, or by any
10 other standard technique.

5.3.4. Biological Peptide Libraries

Biological peptide libraries can be used to express and identify peptides that bind to GIT transport receptors.
15 According to this second approach, involving recombinant DNA techniques, peptides can, by way of example, be expressed in biological systems as either soluble fusion proteins or viral capsid proteins.

20 **5.3.4.1. Methods To Identify Binders:
 Construction Of Libraries**

In a specific embodiment, the peptides of the invention that specifically bind to GIT transport receptors are identified by screening a random peptide library by contacting the library with a ligand selected from among HPT1, hPEPT1, D2H, or hSI (or a molecule consisting
25 essentially of an extracellular domain thereof or fragment of the domain) to identify members of the library that specifically bind to the ligand.

In a particular embodiment, a process to identify the peptides of the present method utilizes a library of recombinant vectors constructed by methods well known in the
30 art and comprises screening a library of recombinant vectors expressing inserted synthetic oligonucleotide sequences

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encoding extracellular GIT transport receptor domains, for example, attached to an accessible surface structural protein of a vector to isolate those members producing peptides that bind to HPT1, hPEPT1, D2H, or hSI. The nucleic acid sequence of the inserted synthetic oligonucleotides of the isolated
5 vector is determined and the amino acid sequence encoded can be deduced to identify a binding domain that binds the ligand of choice (e.g., HPT1, hPEPT1, D2H, or hSI).

The present invention encompasses a method for identifying a peptide which binds to a ligand selected from among HPT1, hPEPT1, D2H, or hSI comprising: screening a
10 library of random peptides with the ligand (or an extracellular domain or fragment thereof) under conditions conducive to ligand binding and isolating the peptide which binds to the ligand. Additionally, the methods of the invention further comprise determining the nucleotide
15 sequence encoding the binding domain of the peptide identified to deduce the amino acid sequence of the binding domain.

5.3.4.2. Preparation of Extracellular Domain Ligand

In a specific embodiment, molecules consisting
20 essentially of an extracellular domain of the desired GIT transport receptor or a fragment of an extracellular domain are used to screen a random peptide library for binding thereto. Preferably, a nucleic acid encoding the extracellular domain is cloned and recombinantly expressed, and the domain is then purified for use. The GIT transport
25 receptor is preferably selected from among HPT1, hPEPT1, D2H, or hSI.

5.3.4.3. Methods to Identify Binders: Screening Libraries

Once a suitable random peptide library has been
30 constructed (or otherwise obtained), the library is screened

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to identify peptides having binding affinity for the GIT transport receptor, e.g., HPT1, hPEPT1, D2H, or hSI. In a preferred aspect, the library is a TSAR library (see U.S. Patent No. 5,498,538 dated March 12, 1996 and PCT Publication WO 94/18318 dated August 18, 1994, both of which are

5 incorporated by reference herein in their entireties). Screening the libraries can be accomplished by any of a variety of methods known to those of skill in the art. See, e.g., the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251: 215-218; Scott and Smith, 1990, Science 249:
10 386-390; Fowlkes et al., 1992; BioTechniques 13: 422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89: 5393-5397; Yu et al., 1994, Cell 76: 933-945; Staudt et al., 1988, Science 241: 577-580; Bock et al., 1992, Nature 355: 564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89:
15 6988-6992; Ellington et al., 1992, Nature 355: 850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; and Rebar and Pabo, 1993, Science 263: 671-673. See also PCT publication WO 94/18318, dated August 18, 1994.

One of ordinary skill in the art will recognize that, with suitable modifications, the screening methods
20 described below would be suitable for a wide variety of biological expression libraries.

Once a library has been constructed or otherwise obtained, the library is screened to identify binding molecules having specific binding affinity for a ligand for a GIT transport receptor preferably selected from among HPT1,
25 hPEPT1, D2H, or hSI.

Screening the libraries can be accomplished by any of a variety of methods known to those of skill in the art. Exemplary screening methods are described in Fowlkes et al., 1992, BioTechniques, 13:422-427 and include contacting the
30 vectors with an immobilized target ligand and harvesting those vectors that bind to said ligand. Such useful

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screening methods, are designated "panning" methods. In panning methods useful to screen the present libraries, the target ligand can be immobilized on plates, beads (such as magnetic beads), sepharose, beads used in columns, etc. If desired, the immobilized target ligand can be "tagged", e.g.,
5 using labels such as biotin, fluorescein isothiocyanate, rhodamine, etc. e.g. for FACS sorting. Panning is also disclosed in Parmley, S.F. and Smith, G.P., 1988, Gene 73: 305-318.

10 In a particular embodiment of the invention, the library can be screened with a recombinant receptor domain. In another embodiment, the library can be screened successively with receptor domains and then on CaCO-2 cells.

For screening of the peptide libraries *in vitro*, the solvent requirements involved in screening are not limited to aqueous solvents; thus, nonphysiological binding
15 interactions and conditions different from those found *in vivo* can be exploited.

Screening a library can be achieved using a method comprising a first "enrichment" step and a second filter lift as follows. The following description is given by way of example, not limitation.

20 Binders from an expressed library (e.g., in phage) capable of binding to a given ligand ("positives") are initially enriched by one or two cycles of panning or affinity chromatography. A microtiter well is passively coated with the ligand (e.g., about 10 μ g in 100 μ l). The
25 well is then blocked with a solution of BSA to prevent non-specific adherence of the phage of the library to the plastic surface. For example, about 10^{11} phage particles expressing peptides are then added to the well and incubated for several hours. Unbound phage are removed by repeated washing of the plate, and specifically bound phage are eluted
30 using an acidic glycine-HCl solution or other elution buffer. The eluted phage solution is neutralized with alkali, and

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developed by exposure to enzyme substrate solution (in the case of directly labeled probe) or further exposed to a solution of enzyme-labeled avidin (in the case of biotinylated probe). Positive phage plaques are identified by localized deposition of colored enzymatic cleavage product
5 on the filter which corresponds to plaques on the original plate. The developed filter is simply realigned with the plate using the registration marks, and the "positive" plaques are cored from the agarose to recover the phage. Because of the high density of plaques on the original plate, it may be difficult to isolate a single plaque from the plate
10 on the first pass. Accordingly, phage recovered from the initial core can be re-plated at low density and the process can be repeated to allow isolation of individual plaques and hence single clones of phage.

Successful screening experiments are optimally conducted using 3 rounds of serial screening. The recovered
15 cells are then plated at a low density to yield isolated colonies for individual analysis. The individual colonies are selected and used to inoculate LB culture medium containing ampicillin. After overnight culture at 37°C, the cultures are then spun down by centrifugation. Individual cell aliquots are then retested for binding to the target
20 ligand attached to the beads. Binding to other beads having attached thereto a non-relevant ligand, can be used as a negative control.

One aspect of screening the libraries is that of elution. The following discussion is applicable to any system where the random peptide is expressed on a surface
25 fusion molecule. It is conceivable that the conditions that disrupt the peptide-target interactions during recovery of the phage are specific for every given peptide sequence from a plurality of proteins expressed on phage. For example, certain interactions may be disrupted by acid pH but not by basic pH, and vice versa. Thus, it may be desirable to test
30 a variety of elution conditions (including but not limited to

pH 2-3, pH 12-13, excess target in competition, detergents, mild protein denaturants, urea, varying temperature, light, presence or absence of metal ions, chelators, etc.) and compare the primary structures of the binding proteins expressed on the phage recovered for each set of conditions
5 to determine the appropriate elution conditions for each ligand/binding protein combination. Some of these elution conditions may be incompatible with phage infection because they are bactericidal and will need to be removed by dialysis (i.e., dialysis bag, Centricon/Amicon microconcentrators).

In a preferred embodiment, a phage display library
10 of random peptides is screened to select phage expressing peptides that bind to a GIT transport receptor. Preferably, a first step is to isolate a preselected phage library. The "preselected phage library" is a library consisting of a subpopulation of a phage display library. This subpopulation can be formed by initially screening against either a target
15 GIT transport receptor (or domain thereof) so as to permit the selection of a subpopulation of phages which specifically bind to the receptor. Alternatively, the subpopulation can be formed by screening against a target cell or cell type or tissue type or tissue barrier of the gastro-intestinal tract, so as to permit the selection of a subpopulation of phages
20 which either bind specifically to the target cell or target cell type or target tissue or target tissue barrier, or which binds to and/or is transported across (or between) the target cell or target cell type or target tissue or target tissue barrier either *in situ* or *in vivo*. This preselected phage
25 library or subpopulation of selected phages can also be rescreened against the target GIT transport receptor, permitting the further selection of a subpopulation of phages which bind to the GIT transport receptor or target cell or target cell type or target tissue or target tissue barrier or which bind to and/or is transported across the target cell,
30 target tissue or target tissue barrier either *in situ* or *in vivo*. Such rescreening can be repeated from zero to 30 times

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with each successive "pre-selected phage library" generating additional pre-selected phage libraries.

In a preferred embodiment, a preselected phage library binding a ligand that is a GIT transport receptor preferably selected from among HPT1, hPEPT1, D2H, or hSI is
5 obtained by an *in vitro* screening step as described above, and then the phage are optionally further characterized using *in vitro* assays consisting of binding phage directly to the receptor domain of interest or, alternatively, to Caco-2 cells or using *in vivo* assays. In another preferred
10 embodiment, *in vivo* assays are used that measure uptake of phage by intestinal tissue or, alternatively, through the GIT. In alternative embodiments, such further *in vitro* or *in vivo* assays can be used as the initial screening step.

In vivo assays that may be used are described in
15 the examples *infra*.

5.4. Generation of Antibodies to GIT Transport Receptor-Binding Peptides and Derivatives Thereof

According to the invention, a GIT transport receptor-binding peptide, fragments or other derivatives, or
20 analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

Various procedures known in the art may be used for
25 the production of polyclonal antibodies to a GIT transport receptor-binding peptide or derivative or analog. For the production of antibody, various host animals can be immunized by injection with the native GIT transport receptor-binding peptides, or a synthetic version, or derivative (e.g., fragment) thereof, including but not limited to rabbits,
30 mice, rats, fowl, etc. Various adjuvants may be used to increase the immunological response, depending on the host

species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human
5 adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward a GIT transport receptor-binding peptide or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be
10 used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal
15 antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci.
20 U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). According to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al.,
25 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for GIT transport receptor-binding peptides together with genes from a human antibody molecule of appropriate biological activity can be used.

According to the invention, techniques described
30 for the production of single chain antibodies (U.S. Patent

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4,946,778) can be adapted to produce GIT transport
receptor-binding peptide-specific single chain antibodies.
An additional embodiment of the invention utilizes the
techniques described for the construction of Fab expression
libraries (Huse et al., 1989, Science 246:1275-1281) to allow
5 rapid and easy identification of monoclonal Fab fragments
with the desired specificity for GIT transport
receptor-binding peptides, derivatives, or analogs.

Antibody fragments which contain the idiotype of
the molecule can be generated by known techniques. For
example, such fragments include but are not limited to: the
10 F(ab')₂ fragment which can be produced by pepsin digestion of
the antibody molecule; the Fab' fragments which can be
generated by reducing the disulfide bridges of the F(ab')₂
fragment, the Fab fragments which can be generated by
treating the antibody molecule with papain and a reducing
agent, and Fv fragments.

15 In the production of antibodies, screening for the
desired antibody can be accomplished by techniques known in
the art, e.g. ELISA (enzyme-linked immunosorbent assay). For
example, to select antibodies which recognize a specific
domain of a GIT transport receptor-binding peptide, one may
assay generated hybridomas for a product which binds to a GIT
20 transport receptor-binding peptide fragment containing such a
domain.

Antibodies specific to a domain of a GIT transport
receptor-binding peptide are also provided.

The foregoing antibodies can be used in methods
known in the art relating to the localization and activity of
25 the GIT transport receptor-binding peptide sequences of the
invention, e.g., for imaging these peptides after *in vivo*
administration (e.g., to monitor treatment efficacy),
measuring levels thereof in appropriate physiological
samples, in diagnostic methods, etc. For instance,
30 antibodies or antibody fragments specific to a domain of a
GIT transport receptor-binding peptide or to a derivative of

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a peptide, such as a dansyl group or some other epitope introduced into the peptide, can be used to 1) identify the presence of the peptide on a nanoparticle or other substrate; 2) quantify the amount of peptide on the nanoparticle; 3) measure the level of the peptide in appropriate
5 physiological samples; 4) perform immunohistology on tissue samples; 5) image the peptide after *in vivo* administration; 6) purify the peptide from a mixture using an immunoaffinity column or 7) bind or fix the peptide to the surface of nanoparticle. This last use envisions attaching the antibody (or fragment of the antibody) to the surface of drug-loaded
10 nanoparticles or other substrate and then incubating this conjugate with the peptide. This procedure results in binding of the peptide in a certain fixed orientation, resulting in a particle that contains the peptide bound to the antibody in such a way that the peptide is fully active.

Abtides (or Antigen binding peptides) specific to a
15 domain of a GIT transport receptor-binding peptide or to a derivative of a peptide, such as a dansyl group or some other epitope introduced into the peptide, can be used for the same seven purposes identified above for antibodies.

20 5.5. Assays of GIT Transport Receptor-Binding Peptides, Derivatives and Analogs

The functional activity of GIT transport receptor-binding peptides, derivatives and analogs can be assayed by various methods.

In a preferred embodiment, in which binding to a
25 GIT transport receptor is being assayed, the binding can be assayed by *in vivo* or *in vitro* assays such as described in the examples *infra*, or by other means that are known in the art.

In another embodiment, where one is assaying for the ability to bind or compete with full-length GIT transport
30 receptor-binding peptide for binding to anti-GIT transport receptor-binding peptide antibody, various immunoassays known

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in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labelled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

Other methods will be known to the skilled artisan and are within the scope of the invention.

20 5.6. Uses

The invention provides compositions comprising the GIT transport receptor-binding proteins of the invention bound to a material comprising an active agent. Such compositions have use in targeting the active agent to the GIT and/or in facilitating transfer through the lumen of the GIT into the systemic circulation. Where the active agent is an imaging agent, such compositions can be administered *in vivo* to image the GIT (or particular transport receptors thereof). Other active agents include but are not limited to: any drug or antigen or any drug- or antigen-loaded or drug- or antigen-encapsulated nanoparticle, microparticle, liposome, or micellar formulation capable of eliciting a biological response in a human or animal. Examples of

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drug-or antigen-loaded or drug- or antigen-encapsulated formulations include those in which the active agent is encapsulated or loaded into nano- or microparticles, such as biodegradable nano- or microparticles, and which have the GIT transport receptor-binding protein or derivative or analog

5 adsorbed, coated or covalently bound, such as directly linked or linked via a linking moiety, onto the surface of the nano-or microparticle. Additionally, the protein, derivative or analog can form the nano- or microparticle itself or the protein, derivative or analog can be covalently attached to the polymer or polymers used in the production of the

10 biodegradable nano- or microparticles or drug-loaded or drug-encapsulated nano- or microparticles or the peptide can be directly conjugated to the active agent. Such conjugations to active agents include fusion proteins in which a DNA sequence coding for the peptide is fused in-frame to the gene or cDNA coding for a therapeutic peptide or

15 protein such that the modified gene codes for a recombinant fusion protein.

In a preferred embodiment, the invention provides for treatment of various diseases and disorders by administration of a therapeutic compound (termed herein "Therapeutic"). Such "Therapeutics" include but are not

20 limited to: GIT transport receptor-binding proteins, and analogs and derivatives (including fragments) thereof (e.g., as described hereinabove) that bind to GIT transport receptors, bound to an active agent of value in the treatment or prevention of a disease or disorder (preferably a mammalian, most preferably human, disease or disorder).

25 Therapeutics also include but are not limited to nucleic acids encoding the GIT transport receptor-binding proteins, analogs, or derivatives bound to such a therapeutic or prophylactic active agent. The active agent is preferably a drug.

Any drug known in the art may be used, depending

30 upon the disease or disorder to be treated or prevented, and

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the type of subject to which it is to be administered. As used herein, the term "drug" includes, without limitation, any pharmaceutically active agent. Representative drugs include, but are not limited to, peptides or proteins, hormones, analgesics, anti-migraine agents, anti-coagulant

5 agents, anti-emetic agents, cardiovascular agents, anti-hypertensive agents, narcotic antagonists, chelating agents, anti-anginal agents, chemotherapy agents, sedatives, anti-neoplastics, prostaglandins, and antidiuretic agents. Typical drugs include peptides, proteins or hormones such as insulin, calcitonin, calcitonin gene regulating protein,

10 atrial natriuretic protein, colony stimulating factor, betaseron, erythropoietin (EPO), interferons such as α , β or γ interferon, somatropin, somatotropin, somatostatin, insulin-like growth factor (somatomedins), luteinizing hormone releasing hormone (LHRH), tissue plasminogen activator (TPA), growth hormone releasing hormone (GHRH),

15 oxytocin, estradiol, growth hormones, leuprolide acetate, factor VIII, interleukins such as interleukin-2, and analogs thereof; analgesics such as fentanyl, sufentanil, butorphanol, buprenorphine, levorphanol, morphine, hydromorphone, hydrocodone, oxymorphone, methadone, lidocaine, bupivacaine, diclofenac, naproxen, paverin, and analogs

20 thereof; anti-migraine agents such as heparin, hirudin, and analogs thereof; anti-coagulant agents such as scopolamine, ondansetron, domperidone, etoclopramide, and analogs thereof; cardiovascular agents, anti-hypertensive agents and vasodilators such as diltiazem, clonidine, nifedipine, verapamil, isosorbide-5-mononitrate, organic nitrates, agents

25 used in treatment of heart disorders and analogs thereof; sedatives such as benzodiazepines, phenothiozines and analogs thereof; narcotic antagonists such as naltrexone, naloxone and analogs thereof; chelating agents such as deferoxamine and analogs thereof; anti-diuretic agents such as desmopressin, vasopressin and analogs thereof; anti-anginal

30 agents such as nitroglycerine and analogs thereof; anti-neoplastics such as 5-fluorouracil, bleomycin and

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analogues thereof; prostaglandins and analogues thereof; and chemotherapy agents such as vincristine and analogues thereof. Representative drugs also include but are not limited to antisense oligonucleotides, genes, gene correcting hybrid oligonucleotides, ribozymes, aptameric oligonucleotides, 5 triple-helix forming oligonucleotides, inhibitors of signal transduction pathways, tyrosine kinase inhibitors and DNA modifying agents. Drugs that can be used also include, without limitation, systems containing gene therapeutics, including viral systems for therapeutic gene delivery such as adenovirus, adeno-associated virus, retroviruses, herpes 10 simplex virus, sindbus virus, liposomes, cationic lipids, dendrimers, and enzymes. For instance, gene delivery viruses can be modified such that they express the targeting peptide on the surface so as to permit targeted gene delivery.

In a preferred embodiment, a Therapeutic is therapeutically or prophylactically administered to a human 15 patient.

Additional descriptions and sources of Therapeutics that can be used according to the invention are found in various Sections herein.

5.7. Therapeutic/Prophylactic Administration, 20 Compositions and Formulations

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to 25 animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably a human.

As will be clear, any disease or disorder of interest amenable to therapy or prophylaxis by providing a drug *in vivo* systemically or by targeting a drug *in vivo* to 30 the GIT (by linkage to a GIT transport-receptor binding

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protein, derivative or analog of the invention) can be treated or prevented by administration of a Therapeutic of the invention. Such diseases may include but are not limited to hypertension, diabetes, osteoporosis, hemophilia, anemia, cancer, migraine, and angina pectoris, to name but a few.

5 Any route of administration known in the art may be used, including but not limited to oral, nasal, topical, intravenous, intraperitoneal, intradermal, mucosal, intrathecal, intramuscular, etc. Preferably, administration is oral; in such an embodiment the GIT-transport binding protein, derivative or analog of the invention acts
10 advantageously to facilitate transport of the therapeutic active agent through the lumen of the GIT into the systemic circulation.

 The present invention also provides therapeutic compositions/formulations. In a specific embodiment of the invention, a GIT transport receptor-binding peptide or motif
15 of interest is associated with a therapeutically or prophylactically active agent, preferably a drug or drug-containing nano- or microparticle. More preferably, the active agent is a drug encapsulating or drug loaded nano- or microparticle, such as a biodegradable nano- or microparticle, in which the peptide is physically adsorbed or
20 coated or covalently bonded, such as directly linked or linked via a linking moiety, onto the surface of the nano- or microparticle. Alternatively, the peptide can form the nano-or microparticle itself or can be directly conjugated to the active agent. Such conjugations include fusion proteins in which a DNA sequence coding for the peptide is fused
25 in-frame to the gene or cDNA coding for a therapeutic peptide or protein, such that the modified gene codes for a recombinant fusion protein in which the "targeting" peptide is fused to the therapeutic peptide or protein and where the "targeting" peptide increases the absorption of the fusion protein from the GIT. Preferably the particles range in size
30 from 200-600 nm.

Thus, in a specific embodiment, a GIT transport-binding protein is bound to a slow-release (controlled release) device containing a drug. In a specific embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)).

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying

agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

5 Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically
10 effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those
15 derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

20 The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage
25 ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances.

30 6. EXAMPLES

6.1. Selection of GIT Receptor Targets

The HPT1, hPEPT1, D2H, and hSI receptors were selected for cloning as GIT receptor targets based on several criteria, including: (1) expression on surface of epithelial cells in gastro-intestinal tract (GIT); (2) expression along
5 the length of small intestine (HPT1, hPEPT1, D2H); (3) expression locally at high concentration (hSI); (4) large putative extracellular domains facing into the lumen of the GIT; and (5) extracellular domains that permit easy access and bioadhesion by targeting particles.

10 The four recombinant receptor sites screened with the peptide libraries additionally have the following characteristics:

| | <u>Receptor</u> | <u>Characteristics</u> |
|----|-----------------|---|
| | D2H | Transport of neutral/basic amino acids; a transport activating protein for a range of amino acid translocases |
| 15 | hSI | Metabolism of sucrose and other sugars; represents 9% of brush border membrane protein in Jejunum |
| | HPT1 | di/tri peptide transporter or facilitator of peptide transport |
| | hPEPT1 | di/tri peptide transporter |

20 Figures 1-4 (SEQ ID NOS:176, 178, 179, and 181, respectively) show the predicted amino acid sequences for hPEPT1, HPT1, hSI and D2H, respectively.

6.2. Cloning of Extracellular Domain of Selected Receptor Site

25 The following receptor domains were cloned and expressed as His-tag fusion proteins by standard techniques:

| | <u>Receptor</u> | <u>Domain (amino acid residues)</u> |
|----|---------------------|-------------------------------------|
| | hPEPT1 ^a | 391-571 |
| | HPT1 ^b | 29-273 |
| 30 | hSI ^c | 272-667 |
| | D2H ^d | 387-685 |

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- 5 ^a Liang et al., 1995, J. Biol. Chem. 270:6456-6463
 ^b Dantzig et al., 1994, Association of Intestinal
 Peptide Transport with a Protein Related to the
 Cadherin Superfamily
 ^c Chantret et al., Biochem. J. 285:915-923
 ^d Bertran et al., J. Biol. Chem. 268:14842-14949

The receptor proteins were expressed as His-tag fusion proteins and affinity purified under denaturing conditions, using urea or guanidine HCl, utilizing the pET His-tag metal chelate affinity for Ni-NTA Agarose (Hochuli,
10 E., Purification of recombinant proteins with metal chelate adsorbent, Genetic Engineering, Principals and Methods (J.K. Setlow, ed.), Plenum Press, NY, Vol. 12 (1990), pp. 87-98).

6.3. Phage Libraries

Three phage DC8, D38, and DC43 libraries expressing
15 N-terminal pIII fusions in M13 were used to identify peptides that bind to the GIT receptors. The D38 and DC43 libraries which are composed of 37 and 43 random amino acid domains, respectively, have been described previously (McConnell et al., 1995, Molecular Diversity, 1:165-176). The DC8 library is similar to the other two except that the random insert is
20 8 amino acids long flanked on each side by a cysteine residue (i.e., CX₈C).

6.4. Biopanning

Three rounds of biopanning on the GIT receptors
25 were performed generally by standard methods (McConnell et al., 1995, Molecular Diversity, 1:165-176), using a mixture of the DC8 (1×10^{10} pfu), D38 and DC43 (1×10^{11} pfu) phage libraries. After each round of panning the percentage of phage recovered was determined. Following the first two rounds of panning, the eluted phage were amplified overnight. Phage from the third pan were plated out and 100 plaques were
30 picked, amplified overnight and screened in an ELISA assay

for binding to the relevant receptor and BSA. After data analysis, phage clones were identified which had high absorbance in the ELISA assay and/or a good ratio of binding to target compared to binding to BSA. The Insulin Degrading Enzyme (IDE) and recombinant human tissue factor (hTF) were used as irrelevant controls. Several variations of the standard panning technique, discussed below, were used. Selection or panning methods followed one of two strategies. The first strategy involved panning the mixed libraries on the specific GIT receptor adsorbed to a solid surface. The second strategy panned the libraries twice against the GIT receptor and then against Caco-2 cells (Peterson and Mooseker, 1992, J. Cell Science 102:581-600), Selection methods are reflected in the clone nomenclature as described below:

S designates the clone was identified by binding to the hS1 receptor domain.

D designates the clone was identified by binding to the D2H receptor domain.

P designates the clone was identified by binding to the PEPT1 receptor domain.

H designates the clone was identified by binding to the HPT-1 receptor domain.

Phage designated Ni are from a solid phase band GIT receptor pan that used the standard procedure with the addition of Ni-NTA Agarose (Qiagen, Chatsworth, CA). Receptor coated plates were blocked with 0.5% BSA/PBS containing 160 μ l Ni-NTA agarose and libraries were panned in the presence of 50 μ l Ni-NTA agarose. The receptor proteins were expressed as His-tag fusions. The His-tag has a high affinity for Ni-NTA Agarose. Blocking the plate and panning in the presence of Ni-NTA agarose minimized phage binding to the His-tag portion of the recombinant receptor.

Phage with the designation AX were eluted with acid and Factor Xa. Phage were first eluted by standard acid elution then Factor Xa (New England Biolabs, Beverly, MA: 1 μ g protease in 300 μ l of 20mM Tris-HCL, 100mM NaCl, 2mM CaCl₂) was

added to the panning plate and incubated 2 hours. Phage from both elution methods were pooled together then plated.

Phage with the designation AB were eluted with acid and base. Phage were eluted first by standard acid elution then 100mM triethylamine pH 12.1 was added to the panning
5 plate for 10 minutes. Phage from both elution methods were pooled together then plated.

C designates panning on receptor followed by Caco-2 cells. First and second round pans were performed on the receptor and the third round pan was on snapwells of Caco-2 cells. DCX11, DCX8 and DCX33 were identified by two pans on
10 D2H receptor, third pan on Caco-2 cells. The third round Factor Xa eluate from the Caco-2 cells was screened by ELISA on D2H, BSA and fixed Caco-2 cells. For HCA3 the first two rounds of panning were performed on the HPT-1 receptor and the third pan was on monolayers cultured on snapwells of Caco-2 cells.

15 Phage designated 5PAX were carried through five rounds of panning after which a number of phage were sequenced prior to screening by ELISA.

6.5. Sequencing of Selected Phage

The amino acid sequence of phage inserts
20 demonstrating a good ratio of binding to receptor domains and/or Caco-2 cells over background BSA binding were deduced from the nucleotide sequence obtained by sequencing (Sequenase®, U.S. Biochemical Corp., Cleveland, OH) both DNA strands of the appropriate region in the viral genome. The
25 third round acid eluate was screened by ELISA on HPT-1, BSA and Caco-2 fixed cells. Phage designated 5PAX were carried through five rounds of panning after which a number of phages were sequenced prior to screening by ELISA.

One well of a 24 well plate was coated with 10 µg/ml of GIT receptor and the plate was incubated overnight at 4°C. The plate was blocked with 0.5 BSA-PBS for one hour.
30 A mixture of the DC8, D38 and DC43 phage libraries was added

to the plate and the plate was incubated for 2 to 3 hours at room temperature on a rotator. After washing the well 10 times with 1% BSA plus 0.05% Tween 20 in PBS, the well was eluted with 0.05M glycine, pH2. The phage was then eluted with 0.2M NaPO₄. The eluted phage was titered on agar plates; 5 the remaining phage was amplified overnight. The next day the amplified phage was added to a second coated plate and the panning procedure was repeated as described above. The eluted phage from the second pan as well as the amplified phage from the first pan was titered on agar plates. Following amplification overnight of the phage from the 10 second pan, the panning procedure was repeated as described above. The phage eluted from the third pan and the amplified phage from the second pan were then titered overnight on agar plates. Isolated phage colonies were amplified overnight prior to use in an ELISA assay.

15 **6.6. Receptor ELISA Procedure**

96 well plates were coated overnight with GIT receptor, BSA and, optionally, IDE (insulin degrading enzyme, an irrelevant His-fusion protein) or hTF. The plates were blocked for one hour with 0.5% BSA-PBS. After clarification, the amplified phage were diluted 1:100 in 1% BSA plus 0.05% 20 Tween 20 in PBS and added to the plates. Following incubation of the plates on a rotator for 1 to 2 hours, the plates were washed 5 times with 1% BSA plus 0.05% Tween 20 in PBS. Dilute anti-M13-HRP conjugate (anti-M13 antibody linked to horse radish peroxidase (HRP)) was added to all the wells and the plate was incubated for one hour on a rotator. After 25 the plates were washed 5 times, as described above, TMB substrate was added to the wells. The plates were read at 650nm absorbance.

RECEPTOR ELISA RESULTS:

Below are the results of ELISA assays which 30 assessed the binding of phage panned on the hSI receptor to

microtiter plates coated with hSI and BSA. Table 1 shows the OD results as well as the ratio of hSI to BSA binding.

Table 1

| PHAGE | hSI | BSA | hSI/BSA |
|--------|-------|-------|---------|
| S15 | 0.478 | 0.053 | 9 |
| S21 | 0.845 | 0.092 | 9 |
| S22 | 0.399 | 0.061 | 7 |
| SNi10 | 0.57 | 0.051 | 11 |
| SNi28 | 0.942 | 0.113 | 8 |
| SNi34 | 0.761 | 0.115 | 7 |
| SNi38 | 0.466 | 0.076 | 6 |
| SNi45 | 0.518 | 0.056 | 9 |
| SNiAX2 | 0.383 | 0.065 | 6 |
| SNiAX6 | 0.369 | 0.056 | 7 |
| SNiAX8 | 0.342 | 0.068 | 5 |
| BLANK | 0.063 | 0.042 | 2 |

Below are the results of an ELISA which assessed the binding of phage panned on the D2H receptor to microtiter plates coated with D2H and BSA. Table 2 shows the OD results as well as the ratio of D2H to BSA binding.

Table 2

| Phage | D2H | BSA | D2H/BSA |
|-------|-------|-------|---------|
| DAB3 | 0.406 | 0.072 | 6 |
| DAB7 | 0.702 | 0.09 | 8 |
| DAB10 | 0.644 | 0.153 | 4 |
| DAB18 | 0.467 | 0.085 | 5 |
| DAB24 | 1.801 | 0.441 | 4 |
| DAB30 | 0.704 | 0.121 | 6 |
| DAX15 | 0.391 | 0.101 | 4 |
| DAX23 | 0.698 | 0.153 | 5 |
| DAX24 | 0.591 | 0.118 | 5 |
| DAX27 | 1.577 | 0.424 | 4 |
| BLANK | 0.038 | 0.037 | 1 |

Below are the results of an ELISA which assessed the binding of phage panned for two rounds on the D2H receptor followed by a third round pan on Caco-2 snapwells. Binding to fixed Caco-2 cells, D2H and BSA was examined.

Table 3 shows the OD results as well as the ratio of D2H to BSA binding.

Table 3

| PHAGE | Caco-2 | D2H | BSA | D2H/BSA |
|-------|--------|-------|-------|---------|
| DCX8 | 0.498 | 0.163 | 0.063 | 3 |
| DCX11 | 0.224 | 0.222 | 0.071 | 3 |
| DCX26 | 0.114 | 0.956 | 0.213 | 4 |
| DCX33 | 0.164 | 0.616 | 0.103 | 6 |
| DCX36 | 0.149 | 0.293 | 0.064 | 5 |
| DCX39 | 0.121 | 0.299 | 0.066 | 5 |
| DCX42 | 0.308 | 0.158 | 0.065 | 2 |
| DCX45 | 0.147 | 0.336 | 0.075 | 4 |
| Blank | 0.065 | 0.043 | 0.04 | 1 |

Below are the results of an ELISA which assessed the binding of phage panned on the hPEPT1 receptor to hPEPT1 and BSA. Table 4 shows the OD results as well as the ratio of hPEPT1 to BSA binding.

Table 4

| PHAGE | hPEPT1 | BSA | PEPT1/BSA |
|-------|--------|-------|-----------|
| PAX9 | 0.312 | 0.079 | 4 |
| PAX14 | 1.102 | 0.139 | 8 |
| PAX15 | 0.301 | 0.079 | 4 |
| PAX16 | 0.648 | 0.171 | 4 |
| PAX17 | 0.514 | 0.095 | 5 |
| PAX18 | 0.416 | 0.087 | 5 |
| PAX35 | 0.474 | 0.065 | 7 |
| PAX38 | 0.292 | 0.064 | 5 |
| PAX40 | 0.461 | 0.076 | 6 |
| PAX43 | 0.345 | 0.069 | 5 |
| PAX45 | 0.419 | 0.081 | 5 |
| PAX46 | 0.429 | 0.077 | 6 |
| P31 | 0.807 | 0.075 | 11 |
| P90 | 1.117 | 0.107 | 9 |

| | | | |
|--------|-------|-------|---|
| 5PAX3 | 0.173 | 0.04 | 4 |
| 5PAX5 | 0.15 | 0.036 | 4 |
| 5PAX7 | 0.171 | 0.037 | 5 |
| 5PAX12 | 0.227 | 0.04 | 6 |
| Blank | 0.102 | 0.039 | 3 |

5

Table 5 shows the results of an ELISA which assessed the binding of phage panned on the HPT-1 receptor to HPT-1 and BSA. The table shows the OD results as well as the ratio of HPT-1 to BSA binding.

Table 5

10

| PHAGE | HPT1 | BSA | HPT/BSA |
|-------|-------|-------|---------|
| HAX9 | 0.382 | 0.075 | 5 |
| HAX40 | 0.991 | 0.065 | 15 |
| HAX42 | 0.32 | 0.071 | 5 |

15

Table 6 shows the results of an ELISA which assessed the binding of phage panned for two rounds on the HPT-1 receptor followed by a third round pan on Caco-2 snapwells. Binding to fixed Caco-2 cells, HPT-1 and BSA was examined. The table shows the OD results as well as the ratio of HPT-1 to BSA binding.

20

Table 6

| PHAGE | Caco-2 | HPT1 | BSA | HPT1/BSA |
|-------|--------|-------|-------|----------|
| HCA3 | 0.406 | 0.048 | 0.038 | 1 |

CELL ELISA PROCEDURE

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Phage ELISA was used as described above with the following changes. Diluent and wash buffer was PBS containing 1%BSA and 0.05% Tween 20 and plates were washed five times at each wash step. Supernatant of infected bacterial cultures was diluted 1:100 and incubated with protein coated plates for 2-3 hours with mild agitation.

30

Anti-M13 Horseradish peroxidase (HRP) conjugate (Pharmacia, Piscataway, NJ) was diluted 1:8000.

Fixed Caco-2, C2BBel, and A431 cell plates were prepared by growing cells on tissue culture treated microtiter plates. When cells were confluent, plates were fixed with 10% formaldehyde, washed twice with PBS and stored with 0.5%BSA-PBS at -20°C. On the day of the assay, thawed
5 plates were treated with PBS containing 0.1% phenylhydrazine for one hour at 37°C followed by two PBS washes and blocking for one hour with 0.5%BSA-PBS. The standard ELISA procedure was followed at this point.

Phage which showed specificity to a GIT receptor was further characterized by ELISA on a variety of
10 recombinant proteins. Phage which continued to exhibit GIT receptor specificity was sequenced.

Table 7

TARGET BINDING PHAGE INSERT SEQUENCES:

| | | SEQ. | |
|----|-------------------|----------------|---|
| | <u>hSI</u> | <u>ID. NO.</u> | |
| 15 | S15 | 1 | RSGAYESPDGRGGRSYVGGGGCGNIGRKHNWGLRTASPACWD |
| | S21 | 2 | SPRSFWPVVSRHESFGISNYLGCYRTCISGMTKSSPIYPRHS |
| | S22 | 3 | SSSSDWGGVPGKVVRERFKGRGCGISITSVLTGKPNPCPEPKAA |
| | SNi10 | 4 | RVGQCTDSDVRRPWARSCAHQGCAGTRNSHGCITRPLRQASAH |
| 20 | SNi28 | 5 | SHSGGMNRAYGDVFRELRDRWNATSHHTRPTPQLPRGPN |
| | SNi34 | 6 | SPCGGSWGRFMQGGFLGGRTDGC GAHRNRTSASLEPPSSDY |
| | SNi38 | 7 | RGAADQRRGWS ENLGLPRVGWDAIAHNSYTFTSRRRPRPP |
| | SNi45 | 8 | SGGEVSSWGRVNDLCARVSWTGC GTARSARTDNKGFLPKHSSLR |
| | SNiAX2 | 9 | SDSDGDHYGLRGGVRC SLRDRGCGLALSTVHAGPPSFYPKLSSP |
| 25 | SNiAX4 | 10 | RSLGNYGVTGTVDVTVLPMPGHANHLGVSSASSSDPPRR |
| | SNiAX6 | 11 | RTTTAKGCLLGSFGVLSGCSFTPTSPPPHLGYPPHSVN |
| | SNiAX8 | 12 | SPKLSSVGVMTKVTELPTEGPNAISIPI SATLGPRNPLR |
| | <u>D2H</u> | | |
| 30 | DAB3 | 13 | RWCGAELCNSVTKKFRPGWRDHANPSTHHRTPPPSQSSP |
| | DAB7 | 14 | RWCGADDPCGASRWRGGNSLFGCGLRCSAAQSTPSGRIHSTSTS |

| | | |
|-------------------|----|---|
| DAB10 | 15 | SKSGEGGDSSRGETGWARVRSHAMTAGRFRWYNQLPSDR |
| DAB18 | 16 | RSSANNCEWKSDWMRRACIARYANSSGPARAVDTKAAP |
| DAB24 | 17 | SKWSWSSRWGSPQDKVEKTRACGGSPSSTNCHPYTFAPPPQAG |
| DAB30 | 18 | SGFWEFSRGLWDGENRKSVRSGCGFRGSSAQGPCVTPATIDKH |
| 5 DAX15 | 19 | SESGRCRSVSRWMTTWQTQKGGCGSNVSRGSPLDPSHQTGHATT |
| DAX23 | 20 | REWRFAGPPLDLWAGPSLPSFNASSHPRALRTYWSQRPR |
| DAX24 | 21 | RMEDIKNSGWRDSCRWGDLRPGCGSRQWYPSNMRRSSRDYPAGGH |
| DAX27 | 22 | SHPWYRHWNHGDFSGSGQSRHTPPESPHGPRPNATI |
| DCX8 | 23 | RYKHDIGCDAGVDKSSSVRGGCGAHSSPPRAGRGRGTMTVSRL |
| 10 DCX11 | 24 | SQGSKQCMQYRTGRLTVGSEYGCGMNPARGHATPAYPARLLPRYR |
| DCX26 | 25 | SGRTTSEISGLWGWGDDRSYGWGNLTPNYIPYRQATNRHRYT |
| DCX33 | 26 | RWNWTVLPATGGHYWTRSTDYHAINNHRPSIPHQHTPI |
| DCX36 | 27 | SWSSWNWSSKTTRLGDRATREGCGPSQSDGCPYNGRLTTVKPRT |
| DCX39 | 28 | SGSLNAWQPRSWVGGAFRSHANNLNPKPTMVTRHPT |
| 15 DCX42 | 29 | RYSGLSPRDNGPACSQEATLEGCGAQRLMSTRRKGRNSRPGWTL |
| DCX45 | 30 | SVGNDKTSRPVSFYGRVSDLWNASLMPKRTTPSSKRHDDG |
| <u>hPEPT1</u> | | |
| PAX9 | 31 | RWPSVGKNGSDTIDVHSNDASTKRSLIYNHRRPLFP |
| 20 PAX14 | 32 | RTFENDGLGVGRSIQKKS DRWYASHNIRSHFASMSPAGK |
| PAX15 | 33 | SYCRVKGGGEGGHTDSNLARSGCGKVARTSRLQHINPRATPPSR |
| PAX16 | 34 | SWTRWGKHTHGGFVNKSPPGKNATSPYTDAQLPDQGP |
| PAX17 | 35 | SQVDSFRNSFRWYEPSRALCHGCGKRDSTSTRIHNSPDSYPTR |
| PAX18 | 36 | SFLRFQSPRFEDYSRTISRLRNATNPSNVSDAHNNRALA |
| 25 PAX35 | 37 | RSITDGGINEVDLSSSVNVLENANSHRAYRKHRPTLKR |
| PAX38 | 38 | SSKVSSPRDPTVPRKGGNVGYGCGHRSSARMPTSALSSITKCYT |
| PAX40 | 39 | RASTQGGRGVAPEFGASVLGRGCGSATYYTNSTSCKDAMGHNYS |
| PAX43 | 40 | RWCEKHKFTAARCSAGAGFERDASRPPQPAHRDNTNRNA |
| PAX45 | 41 | SFQVYPDHGLERHALDGTGPLYAMPGRWIRARPQNRDRQ |
| 30 PAX46 | 42 | SRCTDNEQCPDTGTRSRSVSNARYFSSRLLKTHAPHRP |
| P31 | 43 | SARDSGPAEDGSRAVRLNGVENANTRKSSRSNPRGRRHP |

| | | |
|-----------------|----|---|
| P90 | 44 | SSADAEKCA G S L L W W G R Q N N S G C G S P T K K H L K H R N R S Q T S S S S H |
| 5PAX3 | 45 | R P K N V A D A Y S S Q D G A A A E E T S H A S N A A R K S P K H K P L R R P |
| 5PAX5 | 46 | R G S T G T A G G E R S G V L N L H T R D N A S G S G F K P W Y P S N R G H K |
| 5PAX7 | 47 | R W G W E R S P S D Y D S D M D L G A R R Y A T R T H R A P P R V L K A P L P |
| 5 5PAX12 | 48 | R G W K C E G S Q A A Y G D K D I G R S R G C G S I T K N N T N H A H P S H G A V A K I |

HPT-1

| | | |
|-----------------|----|---|
| HAX9 | 49 | S R E E A N W D G Y K R E M S H R S R F W D A T H L S R P R R P A N S G D P N |
| HAX35 | 50 | E W Y S W K R S S K S T G L G D T A T R E G C G P S Q S D G C P Y N G R L T T V K P R K |
| 10 HAX40 | 51 | R E F A E R R L W G C D D L S W R L D A E G C G P T P S N R A V K H R K P R P R S P A L |
| HAX42 | 52 | S D H A L G T N L R S D N A K E P G D Y N C C G N G N S T G R K V F N R R R P S A I P T |
| HCA3 | 53 | R H I S E Y S F A N S H L M G G E S K R K G C G I N G S F S P T C P R S P T P A F R R T |
| H40 | 54 | S R E S G M W G S W W R G H R L N S T G G N A N M N A S L P D P P V S T P |
| PAX2 | 55 | S T P P S R E A Y S R P Y S V D S D S D T N A K H S S H N R R L R T R S R P N |

15

Table 8

DNA Sequences for Clones used in *in vivo* Pan

S15 (SEQ ID NO: 56)

20 TCTCACTCCTCGAGATCCGGCGCTTATGAGAGTCCGGATGGTCGGGGGGGTCGGAGCTATG
TGGGGGGCGGGGGTGGNTGTGGTAACATTGGTCGGAAGCATAACCTGTGGGGGCTGCGTAC
CGCGTCGCCGGCCTGCTGGGACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

S21 (SEQ ID NO: 57)

TCTCACTCCTCGAGTCCTCGCTCTTTCTGGCCCGTTGTGTCCCGGCATGAGTCGTTTGGGA
TCTCTAACTATTTGGGNTGTGGTTATCGTACATGTATCTCCGGCACGATGACTAAGTCTAG
CCCGATTTACCCTCGGCATTCTGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

25 S22 (SEQ ID NO: 58)

TCTCACTCCTCGAGTAGTAGCTCCGATTGGGGTGGTGTGCCTGGGAAGGTGGTTAGGGAGC
GCTTTAAGGGGCGCGGTTGTGGTATTTCATCACCTCCGTGCTCACTGGGAAGCCCAATCC
GTGTCCGGAGCCTAAGGCGGCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

30

(

SNi 10 (SEQ ID NO: 59)

TCTCACTCCTCGAGAGTTGGCCAGTGCACGGATTCTGATGTGCGGCGTCCTTGGGCCAGGT
CTTGCGCTCATCAGGGTTGTGGTGCGGGCACTCGCAACTCGCACGGCTGCATCACCCGTCC
TCTCCGCCAGGCTAGCGCTCATTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

5 SNi 28 (SEQ ID NO: 60)

TCTCACTCCTCGAGCCACTCCGGTGGTATGAATAGGGCCTACGGGGATGTGTTTAGGGAGC
TTCGTGATCGGTGGAACGCCACTTCCCACCACACTCGCCCCACCCCTCAGCTCCCCCGTGG
GCCTAATTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi 34 (SEQ ID NO: 61)

10 TCTCACTCCTCGAGTCCGTGCGGGGGGTCTGTGGGGGCGTTTTATGCAGGGTGGCCTTTTCG
GCGGTAGGACTGATGGTTGTGGTGCCCATAGAAACCGCACTTCTGCGTCGTTAGAGCCCC
GAGCAGCGACTACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi 38 (SEQ ID NO: 62)

TCTCACTCCTCGAGGGGCGCCGCCGATCAGCGGCGGGGGTGGTCCGAGAACTTGGGGTTGC
CTAGGGTGGGGTGGGACGCCATCGCTCACAATAGCTATACGTTACCTCGCGCCGCCGCG
CCCCCCTCTAGA

15 SNi 45 (SEQ ID NO: 63)

TCTCACTCCTCGAGCGGTGGGGAGGTCAGCTCCTGGGGCCGCGTGAATGACCTCTGCGCTA
GGGTGAGTTGGACTGGTTGTGGTACTGCTCGTTCCGCGCGTACCGACAACAAAGGCTTTCT
TCCTAAGCACTCGTCACTCCGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi AX2 (SEQ ID NO: 64)

20 TCTCACTCCTCGAGTGATAGTGACGGGGATCATTATGGGCTTCGGGGGGGGGTGCGTTGTT
CGCTTCGTGATAGGGGTGTGGTCTGGCCCTGTCCACCGTCCATGCTGGTCCCCCTCTTT
TTACCCCAAGCTCTCCAGCCCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi AX4 (SEQ ID NO: 65)

25 TCTCACTCCTCGAGGAGCTTGGGTAATTATGGCGTCACCGGGACTGTGGACGTGACGGTTT
TGCCCATGCCTGGCCACGCCAACCACCTTGGTGTCTCCTCCGCCTCTAGCTCTGATCCTCC
GCGGCGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi AX6 (SEQ ID NO: 66)

TCTCACTCCTCGAGAACTACGACGGCTAAGGGGTGTCTTCTCGGAAGCTTCGGCGTTCTTA
GTGGGTGCTCATTTACGCCAACCTCTCCACCGCCCCACCTAGGATACCCCCCTCCTCCG
CAATTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

SNi AX8 (SEQ ID NO: 67)

30

TCTCACTCCTCGAGCCCGAAGTTGTCCAGCGTGGGTGTTATGACTAAGGTCACGGAGCTGC
CCACGGAGGGGGCCTAACGCCATTAGTATTCCGATCTCCGCGACCCTCGGCCCCGCGCAACCCGCTCCG

DAB3 (SEQ ID NO: 68)

TCTCACTCCTCGAGGTGGTGCGGCGCTGAGCTGTGCAACTCGGTGACTAAGAAGTTTCGCC
CGGGCTGGCGGGATCACGCCAATCCCTCCACCCATCATCGTACTCCCCCGCCCAGCCAGTC
5 CAGCCCTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DAB7 (SEQ ID NO: 69)

TCTCACTCCTCGAGGTGGTGCGGCGCTGATGACCCGTGTGGTGCCAGTCGTTGGCGGGGGG
GCAACAGCTTGTTTGGTTGTGGTCTTCGTTGTAGTTCGGCGCAGAGCACCCCGAGTGGCAG
GATCCATTCCACTTCGACCAGCTCTAGAATCGAAGGTGCGCTAGACCTTCGAGA

10 DAB10 (SEQ ID NO: 70)

TCTCACTCCTCGAGTAAGTCCGGGGAGGGGGGTGACAGTAGCAGGGGCGAGACGGGGCTGGG
CGAGGGTTTCGGTCTCACGCCATGACTGCTGGCCGCTTTCGGTGGTACAACCAGTTGCCCTC
TGATCGGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DAB18 (SEQ ID NO: 71)

15 TCTCACTCCTCGAGGTCGAGCGCCAATAATTGCGAGTGGAAGTCTGATTGGATGCGCAGGG
CCTGTATTGCTCGTTACGCCAACAGTTCGGGCCCCGCCGCGCCGTCGACACTAAGGCCGC
GCCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DAB24 (SEQ ID NO: 72)

TCTCACTCCTCGAGTAAGTGGTTCGTGGAGTTCGAGGTGGGGCTCCCCGCAGGATAAGGTTG
AGAAGACCAGGGCGGGTTGTGGTGGTAGTCCCAGCAGCACCAATTGTCACCCCTACACCTT
TGCCCCCCCCCGCAAGCCGGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

20 DAB30 (SEQ ID NO: 73)

TCTCACTCCTCGAGTGGGTTCTGGGAGTTTAGCAGGGGGCTTTGGGATGGGGAGAACCGTA
AGAGTGTCCGGTCGGGTTGTGGTTTTTCGTGGCTCCTCTGCTCAGGGCCCGTGTCCGGTCAC
GCCTGCCACCATTGACAAACACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DAX15 (SEQ ID NO: 74)

25 TCTCACTCCTCGAGTGAGAGCGGGCGGTGCCGTAGCGTGAGCCGGTGGATGACGACGTGGC
AGACGCAGAAGGGCGGTTGTGGTTCCAATGTTTCCGCGGTTGCCCCCTCGACCCCTCTCA
CCAGACCGGGCATGCCACTACTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DAX23 (SEQ ID NO: 75)

TCTCACTCCTCGAGGGAGTGGAGGTTTGCCGGGCCCGCTTGGACCTGTGGGCGGGTCCGA
GCTTGCCCTCTTTTAACGCCAGTTCCCACCCTGCGGCCCTGCGCACCTATTGGTCCCAGCG
GCCCCGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

30 DAX24 (SEQ ID NO: 76)

TCTCACTCCTCGAGGATGGAGGACATCAAGAACTCGGGGTGGAGGGACTCTTGTTAGGTGGG
GTGACCTGAGGCCTGGTTGTGGTAGCCGCCAGTGGTACCCCTCGAATATGCGTTCTAGCAG
AGATTACCCCGCGGGGGGCCACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DAX27 (SEQ ID NO: 77)

5 TCTCACTCCTCGAGTCATCCGTGGTACAGGCATTGGAACCATGGTGACTTCTCTGGTTTCGG
GCCAGTCACGCCACACCCCGCCGGAGAGCCCCACCCCGCGCCCTAATGCCACCATTTC
TAGAATCGAAGGTCGCGCTAGACCTTCGAG

DCX8 (SEQ ID NO: 78)

TCTCACTCCTCGAGATATAAGCACGATATCGGTTGCGATGCTGGGGTTGACAAGAAGTCGT
CGTCTGTGCGTGGTGGTTGTGGTGCTCATTNGTCGCCACCCCGCGCCGGCGGTGGTCCTCG
CGGCACGATGGTTAGCAGGCTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

10 DCX11 (SEQ ID NO: 79)

TCTCACTCCTCGAGTCAGGGCTCCAAGCAGTGTATGCAGTACCGCACCGGTCGTTTGACGG
TGGGGTCTGAGTATGGTTGTGGTATGAACCCCGCCCGCCATGCCACGCCCGCTTATCCGGC
GCGCCTGCTGCCACGCTATCGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DCX26 (SEQ ID NO: 80)

15 TCTCACTCCTCGAGTGGGCGGACTACTAGTGAGATTTCTGGGCTCTGGGGTTGGGGTGACG
ACCGGAGCGGTTATGGTTGGGGTAACACGCTCCGCCCCAACTACATCCCTTATAGGCAGGC
GACGAACAGGCATCGTTATACGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DCX33 (SEQ ID NO: 81)

TCTCACTCCTCGAGGTGGAATTGGACTGTCTTGCCCGCCACTGGCGGCCATTACTGGACGC
GTTTCGACGGACTATCACGCCATTAAACAATCACAGGCCGAGCATCCCCACCAGCATCCGAC
CCCTATCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

20 DCX36 (SEQ ID NO: 82)

TCTCACTCCTCGAGTTGGTCGTGCTGGAATTGGAGCTCTAAGACTACTCGTCTGGGCGACA
GGGCGACTCGGGAGGGTTGTGGTCCCAGCCAGTCTGATGGCTGTCCTTATAACGGCCGCCT
TACGACCGTCAAGCCTCGCACGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DCX39 (SEQ ID NO: 83)

25 TCTCACTCCTCGAGTGGTAGTTTGAACGCATGGCAACCGCGGTCATGGGTGGGGGGCGCGT
TCCGGTCACACGCCAACAATAACTTGAACCCCAAGCCCACCATGGTTACTNGTCACCCTAC
CTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

DCX42 (SEQ ID NO: 84)

30 TCTCACTCCTCGAGGTATTTCGGGTTTGTCCCCGCGGGACAACGGTCCCGCTTGTTAGTCAGG
AGGCTACCTTGGAGGGTTGTGGTGCGCAGAGGCTGATGTCCACCCGTCGCAAGGGCCGCAA
CTCCCGCCCCGGGTGGACGCTCTCTAGAATCGAAGGTCGCGCTAGACCCTTCGAGA

DCX45 (SEQ ID NO: 85)

TCTCACTCCTCGAGCGTGGGGAATGATAAGACTAGCAGGCCGGTTTCCTTCTACGGGCGCG
T TAGTGATCTGTGGAACGCCAGCTTGATGCCGAAGCGTACTCCCAGCTCGAAGCGCCACGA
TGATGGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

PAX2 (SEQ ID NO: 86)

5 TCTCACTCCTCGAGTACTCCCCCAGTAGGGAGGCGTATAGTAGGCCCTATAGTGTCGATA
GCGATTTCGGATACGAACGCCAAGCACAGCTCCCACAACCGCCGTNTGCGGACGCGCAGCCG
CCCGAACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

PAX9 (SEQ ID NO: 87)

TCTCACTCCTCGAGATGGCCTAGTGTGGGTTACAAGGGTAATGGCAGTGACACTATTGATG
TTCACAGCAATGACGCCAGTACTAAGAGGTCCCTCATCTATAACCACCGCCGCCCNTCTT
TCCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

10 PAX14 (SEQ ID NO: 88)

TCTCACTCCTCGAGAACGTTTGAGAACGACGGGCTGGGCGTCGGCCGGTCTATTTCAGAAGA
AGTCGGATAGGTGGTACGCCAGCCACAACATTTCGTAGCCATTTTCGCGTCCATGTCTCCCGC
TGGTAAGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

PAX15 (SEQ ID NO: 89)

15 TCTCACTCCTCGAGCTATTGTCTGGGTAAAGGGTGGTGGGGAGGGGGGGCATAACGGATTCCA
ATCTGGCTAGGTCTGGGTGTGGTAAGGTGGCCAGGACCAGCAGGCTTCAGCATATCAACCC
GCGCGCTACCCCCCTCCCGGTCTAGAATCGAAGGTC

PAX16 (SEQ ID NO: 90)

TCTCACTCCTCGAGTTGGACTCGGTGGGGCAAGCACANTCATGGGGGGTTTGTGAACAAGT
CTCCCCCTGGGAAGAACGCCACGAGCCCCTACACCGACGCCAGCTGCCAGTGATCAGGG
TCCTCCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

20 PAX17 (SEQ ID NO: 91)

TCTCACTCCTCGAGTCAGGTTGATTCGTTTCGTAATAGCTTTCGGTGGTATGAGCCGAGCA
GGGCTCTGTGCCATGGTTGTGGTAAGCGCGACACCTCCACCACTCGTATCCACAATAGCCC
CAGCGACTCCTATCCTACACGCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

PAX18 (SEQ ID NO: 92)

25 TCTCACTCCTCGAGCTTTTTGCGGTTCCAGAGTCCGAGGTTTCGAGGATTACAGTAGGACGA
TCTNTCGGTTGCGCAACGCCACGAACCCGAGTAATGTCTCCGATGCGCACAATAACCGGGC
CTTGGCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

PAX35 (SEQ ID NO: 93)

30 TCTCACTCCTCGAGGAGCATACCGACGGGGGCATCAATGAGGTGGACCTGAGTAGTGTGT
CGAACGTTCTTGAGAACGCCAACTCGCATAGGGCCTACAGGAAGCATCGCCCGACCTTGAA
GCGTCCTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

PAX38 (SEQ ID NO: 94)

TCTCACTCCTCGAGTTCGAAGGTGAGCAGCCCGAGGGATCCGACGGTCCCGCGGAAGGGCG
GCAATGTTGATTATGGTTGTGGTCACAGGTCTTCCGCCCGGATGCCTACCTCCGCTCTGTC
GTCGATCACGAAGTGCTACACTTCTAGAATCGAAGGTGCGCGCTAGACCTTCGAGA

PAX40 (SEQ ID NO: 95)

5 TCTCACTCCTCGAGAGCCAGTANGCAGGGCGGCCGGGGTGTGCCCCTGAGTTTGGGGCGA
GCGTTTTGGGTNGTGGTTGTGGTAGCGCCACTTATTACACGAACTCCACCAGCTGCAAGGA
TGCTATGGGCCACAACACTACTCGTCTAGAATCGAAGGTGCGCGNTAGACCTTCGAGA

PAX43 (SEQ ID NO: 96)

TCTCACTCCTCGAGATGGTGCGAGAAGCACAAAGTTTACGGCTGCGCGTTGCAGCGCGGGG
CGGGTTTTGAGAGGGANGCCAGCCGTCCGCCCCAGCCTGCCACCGGGATAATACCAACCG
TAATGCNTNTAGAATCGAAGGTGCGCGCTAGACCTTCGAGA

10 PAX45 (SEQ ID NO: 97)

TCTCACTCCTCGAGTTTTTCAGGTGTACCCGGACCATGGTCTGGAGAGGCATGCTTTGGACG
GGACGGGTCCGCTTTACGCCATGCCCGGCCGCTGGATTAGGGCGCGTCCGCAGAACAGGGA
CCGCCAGTCTAGAATCGAAGGTGCGCGCTAGACCTTCGAGA

PAX46 (SEQ ID NO: 98)

15 TCTCACTCCTCGAGCAGGTGTACGGACAACGAGCAGTGCCCCGATACCGGGANTAGGTCTC
GTTCCGTTAGTAACGCCAGGTACTTTTCGAGCAGGTTGCTCAAGACTCACGCCCCCATCG
CCCTTCTAGAATCGAAGGTGCGCGCTAGACCTTCGAGA

P31 (SEQ ID NO: 99)

TCTCACTCCTCGAGTGCCAGGGATAGCGGGCCTGCGGAGGATGGGTCCCGCGCCGTCCGGT
TGAACGGGGTTGAGAACGCCAACACTAGGAAGTCCTCCCGCAGTAACCCGCGGGGTAGGCG
CCATCCCTCTAGAATCGAAGGTGCGCGCTAGACCTTCGAGA

20 P90 (SEQ ID NO: 100)

TCTCACTCCTCGAGTTCCGCCGATGCGGAGAAGTGTGCGGGCAGTCTGTTGTGGTGGGGTA
GGCAGAACAACTCCGGTTGTGGTTGCGCCACGAAGAAGCATCTGAAGCACCGCAATCGCAG
TCAGACCTCCTCTTCGTCCCCTCTAGAATCGAAGGTGCGCGCTAGACCTTCGAGA

5PAX3 (SEQ ID NO: 101)

25 TCTCACTCCTCGAGACCGAAGAACGTGGCCGATGCTTATTCTGCTCTCAGGACGGGGCGGCGG
CCGAGGAGACGTCTCACGCCAGTAATGCCGCGCGGAAGTCCCCTAAGCACAAAGCCCTTGAG
GCGGCCTTCTAGAATCGAAGGTGCGCGCTAGACCTTCGAGA

5PAX5 (SEQ ID NO: 102)

30 TCTCACTCCTCGAGAGGCAGTACGGGGACGGCCGGCGGCGAGCGTTCCGGGGTGCTCAACC
TGCACACCAGGGATAACGCCAGCGGCAGCGGTTTCAAACCGTGGTACCCTTCGAATCGGGG
TCACAAGTCTAGAATCGAAGGTGCGCGCTAGACCTTCGAGA

5PAX7 (SEQ ID NO: 103)

TCTCACTCCTCGAGGTGGGGGTGGGAGAGGAGTCCGTCCGACTACGATTCTGATATGGACT
TGGGGGCGAGGAGGTACGCCACCCGCACCCACCGCGCGCCCCCTCGCGTCTTGAAGGCTCC
CCTGCCCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

5PAX12 (SEQ ID NO: 104)

5 TCTCACTCCTCGAGGCACTGGAAGTGCGAGGGCTCTCAGGCTGCCTACGGGGACAAGGATA
TCGGGAGGTCCAGGGGTTGTGGTTCCATTACAAAGAATAACACTAATCACGCCCATCCTAG
CCACGGCGCCGTTGCTAAGATCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

HAX9 (SEQ ID NO: 105)

TCTCACTCCTCGAGCCGCGAGGAGGCGAACTGGGACGGCTATAAGAGGGAGATGAGCCACC
GGAGTCGCTTTTGGGACGCCACCCACCTGTCCCGCCCTCGCCGCCCCGCTAACTCTGGTGA
CCCTAACTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

10 HAX40 (SEQ ID NO: 106)

TCTCACTCNTCGAGAGAGTTTCGCGGAGAGGAGGTTGTGGGGGTGTGATGACCTGAGTTGGC
GTCTCGACGCGGAGGGTTGTGGTCCCACTCCGAGCAATCGGGCCGTCAAGCATCGCAAGCC
CCGCCCACGCTCCCCCGCACTCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

HAX42 (SEQ ID NO: 107)

15 TCTCACTCNTNGAGTGATCACGCGTTGGGGACGAATCTGAGGTCTGACAATGCCAAGGAGC
CGGGTGATTACAACCTGTTGTGGTAACGGGAACCTCTACCGGGCGAAAGGTTTTTAACCGTAG
GCGCCCCCTCCGCCATCCCCANTTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

HCA3 (SEQ ID NO: 108)

TCTCACTCCTCGAGGCATATTTCTGAGTATAGCTTTGCGAATTTCCCACTTGATGGGTGGCG
AGTCCAAGCGGAAGGGTTGTGGTATTAACGGCTCCTTTTCTCCCACTTGTCCTCCGCTCCCC
CACCCCAGCCTTCCGCCGCACCTCTAGAATCGAAGGTCGCGCTAGACCTTCGAGA

20 H40 (SEQ ID NO: 109)

TCTCACTCCTCGAGCCGGGAGAGCGGGATGTGGGGTAGTTGGTGGCGTGGTTCACAGGTTGA
ATTCCACGGGGGGTAACGCCAACATGAATGCTAGTCTGCCCCCGACCCCCCTGTTTCCAC
TCCGTCTAGAATCGAAGGTCGCGCTAGACCTTCGAG

Peptide Motifs

25 By comparison of the amino acid sequences of the clones binding GIT receptors, certain sequence similarities or "motifs" were recognized. These motifs can often represent the part of the sequence that is important for binding to the target. Table 9 identifies regions of sequence similarity or sequence motifs (in boldface) that
30 were identified among GIT binding peptides (corresponding SEQ ID NOS. are shown in Table 7).

Table 9

| | PEPT-1 | SEQ. |
|----|--|----------------|
| | HPT1 | ID. NO. |
| | P31 SARDSGPAEDGSRAVRLNGVENAN TRKSSRSNPRGRRHP | 43 |
| | PAX9 RWPSVGKNGSDTIDVHSNDAST KRS LIYNHRRPLFP | 31 |
| 5 | HAX42 SDHALGTNLRSDNAKEPGDYNCCGNGN STGRK -VFNRRRPSAIP T | 52 |
| | PAX2 STPPSREAYSRPYSVSDSD TNAKHSSHNRLR TRSRPN | 55 |
| | hSI | |
| | SNi10 RVGQCTDS DVRRPW ARSCAHQCGAGTRNSHGCITRPLRQASAH | 4 |
| | SNi38 RGAADQ RRGW SENGLPRVGWDAIAHNSYTFTSRRPRPP | 7 |
| | S15 RSGAYESP DGRGGRSY VGGGGCGNIGRKHNLWGLRTAS PACWD | 1 |
| 10 | SNi34 SPCGGSWGRFMQ GGLFG GRTDGC GAHRNRTS ASLEPPSSDY | 6 |
| | D2H | |
| | DAB10 SKSGEGGDSSRGETGWARV RS SHAMTAGR FRWYNQLPSDR | 15 |
| | DAB30 SGFWEFSRGLWDGENRK S VRSGCGFRGSSA QGPCVTPATIDKH | 18 |
| | DCX8 RYKHDIGCDAGVDKKSS SVRGGCG-AHSSP PRAGRGRGT MVSRL | 23 |

15 Phage Binding to Caco-2 Cells

Phage expressing presumed GIT binding peptide inserts were also assayed by ELISA on fixed Caco-2 or C2BBel cells as follows. Cells were plated at 1×10^5 cells/well on 100 μ l culture media and incubated at 30°C in 5% CO₂ overnight. 100 μ l 25% formaldehyde was added to each well for 15 minutes. Contents of the wells were removed by inverting the plate. The plate was then washed 3 times with DPBS. 0.1% phenylhydrazine DPBS solution was added to each well and incubated for 1 hr at 37°C. The plate was inverted and washed 3 times. The plate was blocked with 0.5% BSA-DPBS for 1 hr at room temperature. The plate was inverted and washed 3 times with 1% BPT (PBS containing 1% BSA and 0.05% Tween20). Phage diluted with 1% BPT was added to wells containing fixed cells. Wells without phage added were used to determine background binding of the HRP conjugate. The plates were incubated 2-3 hours on a rotor at room temperature. Plates were washed as before. Plates were incubated with dilute anti-M13-HRP antibody in 1% BPT for 1 hour at room temperature. Following washing, TMB substrate was added and absorbance of the

plates were read at 650 nm. Table 10 shows the relative binding of phage encoding peptides to fixed Caco-2 cells.

Table 10.

5

**Relative binding of phage encoding
peptides to fixed Caco-2 cells**

| | <u>Phage</u> | <u>Fixed Caco-2 cell binding</u> |
|----|--------------|--------------------------------------|
| 10 | SNi10 | ++ |
| | SNi34 | + |
| | P31 | ++ |
| | 5PAX5 | ++ |
| | PAX2 | + |
| | HAX42 | + |
| | DCX8 | +++ |
| | DCX11 | + |
| | H1 | + |
| 15 | M13mpl18 | - |

In vivo phage selection:

Further selection of phage expressing peptides capable of binding to the GIT or transporting the GIT was done as follows. The purified library was resuspended in a buffer, such as TBS or PBS, and introduced onto one side of a tissue barrier, e.g., injected into the duodenum, jejunum, ileum, colon or other *in vivo* animal site using, for instance, a closed loop model or open loop model. Following injection, samples of bodily fluids located across the tissue barrier, e.g., samples of the portal circulation and/or systemic circulation, were withdrawn at predetermined time points, such as 0 to 90 minutes and/or 2 to 6 hours or more. An aliquot of the withdrawn sample (e.g., blood) was used to directly infect a host, e.g., *E. coli*, in order to confirm the presence of phage. The remaining sample was incubated, e.g., overnight incubation with *E. coli* at 37°C with

The purified phage display library (random or preselected) was diluted to 500 μ l in PBS buffer and injected into the closed (or open) intestinal loop model (e.g., rat, rabbit or other species). At time 0 and at successive time points after injection, a sample of either the portal circulation or systemic circulation was withdrawn. An aliquot of the withdrawn blood was incubated with *E. coli*, followed by plating for phage plaques or for transduction units or for colonies where the phage codes for resistance to antibiotics such as tetracycline. The remainder of the withdrawn blood sample (up to 150 μ l) was incubated with 250 μ l of *E. coli* and 5 ml of LB medium or other suitable growth medium. The *E. coli* cultures were incubated overnight by incubation at 37°C on a shaking platform. Blood samples taken at other time points (such as 15 min, 30 min, 45 min, 60 min, up to 6 hours) were processed in a similar manner, permitting amplification of phages present in the portal or systemic circulation in *E. coli* at these times.

Following amplification, the amplified phage was recovered by PEG precipitation and resuspended in PBS buffer or TBS buffer. The titer of the amplified phage, before and after PEG precipitation, was determined. The amplified, PEG precipitated phage was diluted to a known phage titer (generally between 10^8 and 10^{10} phage or plaque forming units (p.f.u.) per ml) and was injected into the GIT of the animal closed (or open) loop model. Blood samples were collected from portal and/or systemic circulation at various time points and the phage transported into the blood samples were amplified in *E. coli* as given above for the first cycle. Subsequently, the phage was PEG-precipitated, resuspended, titered, diluted and injected into the GIT of the animal closed (or open) loop model. This procedure of phage injection followed by collection of portal and/or systemic blood samples and amplification of phage transported into these blood samples can be repeated, for example, up to 10 times, to permit the selection of phages which are preferentially transported from the GIT into the portal and/or systemic circulation.

6.7. Transport of Phage From Rat Lumen Into the Portal and Systemic Circulation

Phage from random phage display libraries as well as control phage were injected into the lumen of the rat gastro-intestinal tract (*in situ* rat closed loop model). Blood
5 was collected over time from either the systemic circulation or portal circulation and the number of phage which were transported to the circulation was determined by titering blood samples in *E. coli*.

The phage display libraries used in this study were D38
10 and DC43 in which gene III codes for random 38-mer and 43-mer peptides, respectively. As a negative control, the identical phage M13mp18, in which gene III does not code for a "random" peptide sequence, was used. Both the library phages D38 and DC43 were prepared from *E. coli*, mixed together, dialyzed against PBS, precipitated using PEG/NaCl and were resuspended in PBS buffer.
15 The M13mp18 control was processed in a similar manner. The titer of each phage sample was determined and the phage samples were diluted in PBS to approximately the same titers prior to injection into the rat closed loop model.

For sampling from the systemic circulation, approximately 15 cm of the duodenum of Wistar rats was tied off
20 (closed loop model), approximately 0.5ml of phage solution was injected into the closed loop and blood (0.4ml) was sampled from the tail vein at various times. The time points used (in min) were: 0, 15, 30, 45, 60, 90, 120, 180, 240 and 300 minutes. For sampling from the portal circulation, the portal vein was catheterized, approximately 15 cm of the duodenum was tied off
25 (closed loop model), 0.5ml of phage solution was injected into the closed loop and blood was sampled from the portal vein catheter at various times. As the portal sampling is delicate, sampling times were restricted to 15, 30, 45 and 60 minutes, where possible. The volume of phage injected into each animal was as follows:

30

| ANIMALS (15) | VOLUME OF PHAGE INJECTED |
|--------------|--------------------------|
| R1-R3 | 0.50 ml |
| R4 | 0.43 ml |
| R5-R15 | 0.45 ml |

The estimated number of transported phage has been adjusted to
 5 account for differences in volume injected into each animal
 (using 0.5 ml as the standard volume).

To investigate transport into the systemic circulation,
 animals R1, R2 and R3 received the control phage M13mp18 and
 animals R4, R5, R6 and R7 received the test phage D38/DC43 mix.
 To investigate transport into the portal circulation, animals R8,
 10 R9 and R10 received the control phage M13mp18 and animals R11,
 R12, R13 and R14 received the test phage D38/DC43 mix. Animal
 R15* received the combined phage samples from animals R4-R7 (see
 Table 11) which were sampled from the systemic circulation on day
 one, followed by amplification in *E. coli*, PEG precipitation and
 resuspension in PBS. On subsequent analysis, the titer of this
 15 phage was found to be 100 times greater than the other phage
 samples used for animals R8-R14. Thus, the data presented for
 animal R15* is adjusted down.

Approximately 0.4 ml of the blood was collected at each
 time point in each model system. 30 μ l of the collected blood
 (systemic) was mixed with 100 μ l of the prepared
 20 *E. coli* strain K91Kan, incubated at 37°C for 30 min, and plated
 out for plaque formation using Top Agarose on LB plates. Various
 negative controls were included in the titering experiments. The
 following day, the number of plaque forming units was determined.
 Similarly, 30 μ l of the collected blood (portal) and serial
 dilutions (1:100, 1:1000) thereof was mixed with 100 μ l of the
 25 prepared *E. coli* strain K91Kan, incubated at 37°C for 30 min, and
 plated out for plaque formation using Top Agarose on LB plates.
 The following day, the number of plaque forming units was
 determined.

In addition, approximately 300 μ l of the collected blood
 from each time point (systemic and portal) was incubated with 5ml
 30 of prepared *E. coli* strain K91Kan in modified growth media

(

containing 5mM MgCl₂/MgSO₄ at 37°C overnight with shaking (to permit phage amplification). The samples were centrifuged and the cell pellet was discarded. Samples of the phage supernatant were collected, serially diluted (10^{-2} , 10^{-4} , 10^{-6} , 10^{-8}) in TBS buffer, and plated for plaques in order to determine the number
5 of plaque forming units present in the amplified phage samples.

Furthermore, an aliquot of phage was removed from the "amplified" supernatants obtained from test animals R4-R7 (samples from each time point were used), combined, and precipitated using PEG for two hours. The precipitated phage was resuspended in PBS buffer and was injected into closed loop model
10 of animal R15*, followed by portal sampling.

The number of phage transported from the closed loop model into the systemic circulation is presented in Table 11 hereafter. The number of phage transported from the closed loop model into the portal circulation is presented in Table 12 hereafter. These numbers are corrected for phage input
15 difference and for volume input differences. Clearly, more phage are present in the portal samples than in the systemic samples, indicative of either hepatic or RES clearance and/or phage instability in the systemic circulation. In addition, the uptake of phage from the GIT into the portal circulation is quite rapid, with substantial number of phages detected within 15 minutes. The
20 results from the portal sampling experiments would also indicate that the kinetics of uptake of phage from the D38/DC43 libraries is quicker than that of the control phage. Thus, there may be preferential uptake of phage coding for random peptide sequences from the GIT into the portal circulation. In the case of animals R13, R14 and R15*, the % of the phage transported into the
25 titrated blood sample within the limited time frame (30, 45 and 15 mins, respectively) was estimated as 0.13%, 1.1% and 0.013%, respectively.

TABLE 11

**NUMBER OF PHAGE TRANSPORTED FROM THE CLOSED
LOOP MODEL INTO THE SYSTEMIC CIRCULATION**

| 5 | Time (min) | R1 | R2 | R3 | R4 | R5 | R6 | R7 |
|----|-----------------------------------|-----|----|-----|----|-----|----|----|
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 15 | 0 | 1 | 9 | 0 | 0 | 1 | 7 |
| | 30 | 2 | 1 | 0 | 0 | 46 | 1 | 11 |
| | 45 | 10 | 4 | 2 | 1 | 32 | 0 | 20 |
| 10 | 60 | 63 | 19 | 21 | 1 | 114 | 0 | 21 |
| | 90 | 104 | 20 | 18 | 3 | 115 | 0 | 22 |
| | 120 | 94 | 24 | 27 | 0 | 64 | 0 | 6 |
| | 180 | 94 | 12 | 23 | 1 | 413 | 0 | 0 |
| | 240 | 14 | 1 | 20 | 0 | 36 | 0 | 0 |
| | 300 | 1 | 1 | 4 | 2 | 0 | 0 | 0 |
| | Total number of transported phage | 382 | 83 | 124 | 8 | 820 | 2 | 87 |

15 Animals R1, R2 and R3 received the control phage
M13mp18.

 Animals R4, R5, R6 and R7 received the test phage
D38/DC43 mix.

Table 12

20

**NUMBER OF PHAGE TRANSPORTED FROM THE CLOSED
LOOP MODEL INTO THE PORTAL CIRCULATION**

| 25 | Time (min) | R8 | R9 | R10 | R11 | R12 | R13 | R14 | R15* |
|----|------------|----|----|-----|-----|---------|---------|-----------|--------|
| | 15 | 15 | 6 | 3 | 1 | 19 | 231,000 | 1,000,000 | 20,000 |
| | 30 | 1 | 5 | 26 | - | 0 | 60,000 | 272,000 | - |
| | 45 | - | 1 | 555 | - | 1 | - | 1,240,000 | - |
| | 60 | - | - | - | - | 420,000 | - | - | - |

 Animals R8, R9 and R10 received the control phage
M13mp18.

30 Animals R11, R12, R13 and R14 received the test phage
D38/DC43 mix.

((

Animal R15* received the combined phage samples from animals R4-R7 (see Table 11) which were sampled from the systemic circulation on day one, followed by PEG precipitation and resuspension in PBS. On subsequent analysis, the titer of this phage was found to be 100 times greater than the other phage
5 samples used for animals R8-R14. Thus, the data measuring phage transport into the portal circulation for animal R15* is adjusted down.

These studies demonstrated that both the control phage and the D38/DC43 phages are transported over time from the lumen of the GIT into the portal and systemic circulation, as
10 demonstrated by titering the phage transported to the blood in *E. coli*. More phage were transported from the test phage samples into the portal circulation than the corresponding control phage sample. In addition, the kinetics of transport of the test phage into the portal circulation appeared to exceed that of the
15 control phage. Phage from the D38/DC43 libraries which appeared in the systemic circulation of different animals (R4-R7) were pooled, amplified in *E. coli*, precipitated, and re-applied to the lumen of the GIT, followed by collection in the portal circulation and titering in *E. coli*. These selected phage were
20 also transported from the lumen of the GIT into the portal circulation. This *in situ* loop model may represent an attractive screening model in which to identify peptide sequences which facilitate transport of phage and particles from the GIT into the circulation.

Using this screening model system, a number of preselected phage libraries now exist, including a one pass
25 systemic phage library from animals R4-R7, a one-pass portal library from animals R11-R14, and a two pass, rapid transport, systemic-portal phage library SP-2 from animal R15*.

30 **6.8. Transport of Phage From Preselected Phage Libraries
From the Rat Lumen Into the Portal and Systemic
Circulation**

Four preselected phage libraries, GI-D2H, GI-hSI, GI-HPT1 and GI-hPEPT1, were constructed by pooling phage previously selected by screening random phage display libraries D38 and DC43 using the HPT1, HPEPT1, D2H and hSI receptor or binding sites located in the GIT. The phage pools, preselected phage libraries
5 are shown in Table 13. Note that the sequences for PAX2, HAX1, HAX5, HAX6, HAX10, H10 and HAX44 are the same. Also, the sequence for HAX40 is the same as that for H44. The corresponding SEQ ID NOS. are shown in Table 7.

Table 13

10

PRESELECTED PHAGE LIBRARIES

| | <u>D2H</u> | <u>HSI</u> | <u>HPT1</u> | <u>hPEPT1</u> |
|----|------------|------------|-------------|---------------|
| | DAB3 | S15 | HAX9 | PAX2 (H10) |
| | DAB7 | S21 | HAX35 | PAX9 |
| | DAB10 | S22 | HAX40 (H44) | PAX14 |
| | DAB18 | SNi10 | HAX42 | PAX15 |
| | DAB24 | SNi28 | HCA3 | PAX16 |
| 15 | DAB30 | SNi34 | HAX1 | PAX17 |
| | DAX15 | SNi38 | HAX5 | PAX18 |
| | DAX23 | SNi45 | HAX6 | PAX35 |
| | DAX24 | SNiAX2 | HAX10 | PAX38 |
| | DAX27 | SNiAX6 | H40 | PAX40 |
| | DCX8 | SNiAX8 | M13mp18 | PAX43 |
| | DCX11 | M13mp18 | | PAX45 |
| | DCX26 | | | PAX46 |
| | DCX33 | | | P31 |
| 20 | DCX36 | | | P90 |
| | DCX39 | | | 5PAX3 |
| | DCX42 | | | 5PAX5 |
| | DCX45 | | | 5PAX7 |
| | M13mp18 | | | 5PAX12 |
| | | | | H40 |
| | | | | M13mp18 |

25 Similar to methods described herein above, these preselected phage libraries together with the negative control phage M13mp18 were injected into the rat closed loop model (6 animals per preselected phage library), blood was collected over time from the portal circulation via the portal vein and, at the termination of the experiment, a systemic blood sample was
30 collected from the tail vein and the intestinal tissue region from the closed loop was collected.

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In particular, phages selected *in vitro* to each receptor or binding site located in the GIT were amplified in *E. coli*, PEG-precipitated, resuspended in TBS and the titer of each phage sample was determined by plaquing in *E. coli* as described
5 above. Subsequently, an equal number of each phage (8×10^8 phage) for each receptor site was pooled into a preselected phage library together with the negative control phage M13mp18 and each preselected phage library was administered to 6 Wistar rats per library (rats 1-6; GI-D2H, rats 7-12; GI-hSI, rats 13-18; GI-hPEPT1, and rats 19-24; GI-HPT1). Using the *in situ* loop
10 model described above, 0.5 ml of preselected phage library solution was injected into the tied-off portion of the duodenum/jejunum. Blood was collected into heparinized tubes from the portal vein at 0, 15, 30, 45 and 60 minutes. A blood sample was taken from the systemic circulation at the end of the experiment. Similarly, the portion of the duodenum/jejunum used
15 for phage injection was taken at the end of the experiment.

Thirty microliters of the collected portal blood (neat and 10^{-2} , 10^{-4} , 10^{-6} dilutions) was added to 30 μ l *E. coli* K91Kan cells (overnight culture) and incubated at 37°C for 10 min. Subsequently, 3 ml of top agarose was added and the samples were plated for plaques. One hundred microliters of the collected
20 portal blood was added to 100 μ l of *E. coli* K91Kan. Five milliliters of LB medium was then added and the samples were incubated at 37°C overnight in a rotating microbial incubator. The *E. coli* was removed by centrifugation and the amplified phage supernatant samples were either titered directly or were
25 PEG-precipitated, resuspended in TBS and titered. Following titration of the amplified phage, samples containing phage from each set of animals were combined, adjusting the titer of each sample to the same titer, and were plated for plaques on LB agar plates (22cm² square plates). Either 12,000 or 24,000 phage were plated for plaques.

30 Thirty microliters of the collected systemic blood (neat and 10^{-2} , 10^{-4} , 10^{-6} dilutions) was added to *E. coli* K91Kan

cells, incubated at 37°C for 10 min. Three ml of top agarose was then added and the samples were plated for plaques. One hundred microliters of the collected systemic blood was added to 100μl of *E. coli* K91Kan, incubated at 37°C for 10 min. Five milliliters of LB medium was then added and the samples were incubated at 37°C overnight in a rotating microbial incubator. The *E. coli* was removed by centrifugation and the amplified phage supernatant samples were either titered directly or were PEG-precipitated, resuspended in TBS and titered. Following titration of the amplified phage, samples containing phage from each set of animals were combined, adjusting the titer of each sample to the same titer, and were plated for plaques on LB agar plates (22cm² square plates). Either 12,000 or 24,000 phage were plated for plaques.

The intestinal tissue portion used in each closed loop was excised. The tissue was cut into small segments, followed by 3 washings in sterile PBS containing protease inhibitors, and homogenized in an Ultra thorex homogeniser (Int-D samples). Alternatively, the tissue (in PBS supplemented with protease inhibitors) was homogenized in an Ultra Thorex homogenizer, washed 3 times in PBS containing protease inhibitors and resuspended in PBS containing protease inhibitors (Int-G samples). In each case, serial dilutions (neat and 10⁻², 10⁻⁴, 10⁻⁶ dilutions) of the tissue homogenate was titered in *E. coli*. In addition, an aliquot (100μl) of the tissue homogenate was added to 100μl of *E. coli* K91Kan, incubated at 37°C for 10 min, followed by addition of 5ml of LB medium and incubation overnight at 37°C in a rotating microbial incubator.

The phage amplified from the portal blood, systemic blood and intestinal tissue was plated for plaques. The plaques were transferred to Hybond-N Nylon filters, followed by denaturation (1.5M NaCl, 0.5M NaOH), neutralization (0.5M TRIS-HCl, pH7.4, 1.5M NaCl), and washing in 2X SSC buffer. The filters were air-dried, and the DNA was cross-linked to the filter (UV crosslinking: 2min, high setting). The filters were

incubated in pre-hybridization buffer (6X SSC, 5X Denhardt's solution, 0.1% SDS, 20µg/ml yeast tRNA) at 40°C-45°C for at least 60 min.

Synthetic oligonucleotides, (22-mers), complimentary to regions coding for the receptor or binding sites used to create the preselected phage library, were synthesized (see Table 14 below).

Table 14

OLIGONUCLEOTIDES USED IN IN VIVO SCREEN

| | CLONE NAME | OLIGO | SEQ. ID. NO. |
|----|------------|--|-----------------|
| 10 | S15 | 5' TCCGGACTCTCATAAGCGCCGG ^{3'} | 111 |
| | S21 | 5' ACAACGGGCCAGAAAGAGCGAG ^{3'} | 112 |
| | S22 | 5' ACACCACCCCAATCGGAGCTAC ^{3'} | 113 |
| | SNi10 | 5' TCAGAATCCGTGCACTGGCCAA ^{3'} | 114 |
| | SNi28 | 5' GCCCTATTTCATACCACCGGAGT ^{3'} | 115 |
| | SNi34 | 5' CATCAGTCCTACCGCCGAAAAG ^{3'} | 116 |
| | SNi38 | 5' CGTATAGCTATTGTGAGCGATG ^{3'} | 117 |
| | SNi45 | 5' ACGCGCGGAACGAGCAGTACCA ^{3'} | 118 |
| 15 | SNiAX2 | 5' CCATAATGATCCCCGTCCTAT ^{3'} | 119 |
| | SNiAX6 | 5' AGACACCCCTTAGCCGTCGTAG ^{3'} | 120 |
| | SNiAX8 | 5' AGCTCCGTGACCTTAGTCATAA ^{3'} | 121 |
| | DAB3 | 5' TGCACAGCTCAGCGCCGCACCA ^{3'} | 122 |
| | DAB7 | 5' ACGGGTCATCAGCGCCGCACCA ^{3'} | 123 |
| | DAB10 | 5' TGTCAACCCCCCTCCCCGGACTT ^{3'} | 124 |
| | DAB18 | 5' ACTCGCAATTATTGGCGCTCGA ^{3'} | 125 |
| | DAB24 | 5' GTCTTCTCAACCTTATCCTGCG ^{3'} | 126 |
| | DAB30 | 5' AAAGCCCCCTGCTAAACTCCCA ^{3'} | 127 |
| 20 | DAX15 | 5' CTGCGTCTGCCACGTCGTCATC ^{3'} | 128 |
| | DAX23 | 5' GTTAAAAGAGGGCAAGCTCGGA ^{3'} | 129 |
| | DAX24 | 5' CCGAGTTCTTGATGTCCTCCAT ^{3'} | 130 |
| | DAX27 | 5' TCCAATGCCTGTACCACGGATG ^{3'} | 131 |
| | DCX8 | 5' TCGCAACCGATATCGTGCTTAT ^{3'} | 132 |
| | DCX11 | 5' TGCATACACTGCTTGGAGCCCT ^{3'} | 133 |
| | DCX26 | 5' GAAATCTCACTAGTAGTCCGCC ^{3'} | 134 |
| | DCX33 | 5' GCGGGCAAGACAGTCCAATTCC ^{3'} | 135 |
| | DCX36 | 5' GAGCTCCAATTCCACGACGACC ^{3'} | 136 |
| 25 | DCX39 | 5' GGTTGCCATGCGTTCAAACCTAC ^{3'} | 137 |
| | DCX42 | 5' TCCCGCGGGGACAAACCCGAAT ^{3'} | 138 |
| | DCX45 | 5' CTGCTAGTCTTATCATTTCCCA ^{3'} | 139 |
| | PAX2 | 5' CTATCGACACTATAGGGCCTAC ^{3'} | 140 |
| | PAX9 | 5' TACCCTTGTAACCCACACTAGG ^{3'} | 141 |
| | PAX14 | 5' TTCTTCTGAATAGACCGGCCGA ^{3'} | 142 |
| | PAX15 | 5' CCACCACCCTTAACCCGACAAT ^{3'} | 143 |
| | PAX16 | 5' AGGGGGAGACTTGTTTCAAAAC ^{3'} | 144 |
| | PAX17 | 5' CGGCTCATACCACCGAAAGCTA ^{3'} | 145 |
| 30 | PAX18 | 5' ATCGTCCTACTGTAATCCTCGA ^{3'} | 146 |
| | PAX35 | 5' GACACACTACTCAGGTCCACCT ^{3'} | 147 |

| | CLONE NAME | OLIGO | SEQ. ID. NO. |
|----|----------------|--|-----------------|
| | PAX38 | 5' CCATAATCAACATTGCCGCCCT ^{3'} | 148 |
| | PAX40 | 5' CAAAACGCTCGCCCCAAACTCA ^{3'} | 149 |
| | PAX43 | 5' GTAAACTTGTGCTTCTCGCACC ^{3'} | 150 |
| | PAX45 | 5' CCATGGTCCGGGTACACCTGAA ^{3'} | 151 |
| | PAX46 | 5' GTTACTAACGGAACGAGACCTA ^{3'} | 152 |
| 5 | P31 | 5' TGTGCGGCTTCTCAACCCCGTT ^{3'} | 153 |
| | P90 | 5' ACAACCGGAGTTGTTCTGCCTA ^{3'} | 154 |
| | 5PAX3 | 5' TAAGCATCGGCCACGTTCTTCG ^{3'} | 155 |
| | 5PAX5 | 5' TTATCCCTGGTGTGCAGGTTGA ^{3'} | 156 |
| | 5PAX7 | 5' TATCAGAATCGTAGTCGGACGG ^{3'} | 157 |
| | 5PAX12 | 5' CTTTGTAATGGAACCACAACCC ^{3'} | 158 |
| | HAX9 | 5' CGGTGGCTCATCTCCCTCTTAT ^{3'} | 159 |
| | HAX35 | 5' ATCAGACTGGCTGGGACCACAA ^{3'} | 160 |
| | HAX40 | 5' CACAACCTCCTCTCCGCGAACT ^{3'} | 161 |
| 10 | HAX42 | 5' AGATTTCGTCCCCAACGCGTGAT ^{3'} | 162 |
| | HCA3 | 5' GGGGAATTCGCAAAGCTATACTC ^{3'} | 163 |
| | H40 | 5' CCCCCTGGAATTCAACCTGTGA ^{3'} | 164 |
| | M13 (positive) | 5' GTCGTCTTTCCAGACGT ^{3'} | 165 |
| | M13 (negative) | 5' CTTGCATGCCTGCAGGTCGAC ^{3'} | 166 |

The oligonucleotides (5pmol) were 5'end labelled with ³²P-ATP and T4 polynucleotide kinase and approximately 2.5pmol of labelled oligonucleotide was used in hybridization studies.

Hybridizations were performed at 40-45°C overnight in buffer containing 6X SSC, 5X Denhardt's solution, 0.1% SDS, 20µg/ml yeast tRNA and the radiolabeled synthetic oligonucleotide, followed by washings (20-30 min at 40-45°C) in the following buffers: (i) 2X SSC / 0.1% SDS, (ii) 1X SSC / 0.1% SDS, (iii) 0.1X SSC / 0.1% SDS. The filters were air-dried and exposed for autoradiography for 15 hours, 24 hours or 72 hours.

Hybridization data indicated that all the oligonucleotide probes bound specifically to their phage target except for the HAX9 probe which apparently was not labeled. A negative control probe that hybridized only to M13mp18 DNA showed a weak to negative signal in all samples tested (data not shown).

Hybridization data for pools from each receptor group of rats was compiled. Tables 15, 16, 17 and 18 show a representative compilation of autoradiograph signals of the HSI, D2H, HPT1 and hPEPT1 receptor groups. These Tables show the phage absorption and uptake from the closed loop GIT model to portal and systemic circulation and phage

absorption/internalization to intestinal tissue. In these Tables, Int-G refers to intestinal tissue homogenized prior to washing and recovery while Int-D refers to intestinal tissue washed prior to homogenization and phage recovery. In all cases, leading phage candidates were present in more than one animal.

5

Table 15

SUMMARY OF AUTORADIOGRAPH SIGNALS OF HSI ANIMAL STUDY

10

| Phage | Portal | Int.-G | Int.-D |
|---------|---------|--------|--------|
| S15 | ++ | +/- | +/- |
| S21 | - | - | - |
| S22 | - | -/+ | - |
| SNi-10 | +++ / + | ++ | ++ |
| SNi-28 | - | - | - |
| SNi-34 | ++ | - | - |
| SNi-38 | ++ | - | - |
| SNi-45 | - | - | - |
| SNiAX-2 | - | - | - |
| SNiAX-6 | - | - | - |
| SNiAX-8 | - | - | - |
| M13 | ++++++ | ++++++ | ++++++ |
| M13 | nd* | + | - |

15

*not detected

20

25

30

Table 16

SUMMARY OF AUTORADIOGRAPH SIGNALS OF D2H ANIMAL STUDY

| | Phage | Portal | Int.-G | Int.-D |
|----|---------|--------|--------|--------|
| 5 | DAB3 | +++ | +/- | -/+ |
| | DAB7 | ++ | ++ | -/+ |
| | DAB10 | ++++++ | +/- | -/+ |
| | DAB18 | - | - | - |
| | DAB24 | - | - | - |
| | DAB30 | ++++ | ++ | +++ |
| | DAX15 | - | - | - |
| | DAX23 | -/+ | + | -/+ |
| | DAX24 | - | - | - |
| 10 | DAX27 | - | + | - |
| | DCX8 | +++++ | +/- | - |
| | DCX11 | ++++++ | ++ | -/+ |
| | DCX26 | - | - | - |
| | DCX33 | +++ | ++ | ++ |
| | DCX36 | - | - | - |
| | DCX39 | - | -/+ | - |
| | DCX42 | - | - | -/+ |
| | DCX45 | - | ++ | - |
| 15 | M13 (+) | +++++ | +++++ | +++++ |
| | M13 (-) | +/- | -/+ | - |

Table 17

SUMMARY OF AUTORADIOGRAPH SIGNALS OF HPT1 ANIMAL STUDY

| | Phage | Int.-G | Portal | Systemic |
|----|---------|--------|--------|----------|
| 20 | H40 | - | - | ++++ |
| | HAX9 | ND | ND | ND |
| | HAX35 | - | + | - |
| | HAX40 | - | - | - |
| | HAX42 | - | ++ | ++ |
| | HCA3 | - | - | - |
| | PAX2 | - | +++ | ++++ |
| 25 | M13 (+) | ++++++ | ++++++ | ++++++ |
| | M13 (-) | - | --/+ | - |

30

Table 18

SUMMARY OF AUTORADIOGRAPH SIGNALS OF hPEPT1 ANIMAL STUDY

| | Phage | Int.-G | Portal | Systemic |
|----|---------|--------|--------|----------|
| 5 | PAX2 | - | ++ | - |
| | PAX9 | ++ | +++ | - |
| | PAX14 | - | ++ | - |
| | PAX15 | -/+ | - | - |
| | PAX16 | - | - | - |
| | PAX17 | + | ++/+ | - |
| | PAX18 | - | - | - |
| | PAX35 | - | - | - |
| | PAX38 | -/+ | - | - |
| 10 | PAX40 | + | +++ | - |
| | PAX43 | + | - | - |
| | PAX45 | - | - | - |
| | PAX46 | - | +++ | - |
| | P31 | ++ | ++++ | ++ |
| | 5PAX3 | ++/+ | ++ | - |
| | 5PAX5 | - | - | ++ |
| | 5PAX7 | +++ | - | - |
| | 5PAX12 | ++++ | ++ | - |
| 15 | H40 | ++ | ++ | - |
| | M13 (+) | ++++++ | ++++++ | ++++++ |
| | M13 (-) | - | - | - |

Apart from the synthetic oligonucleotide to HAX9, all oligonucleotides were initially confirmed to be radiolabeled, as determined by hybridization to the corresponding phage target (eg., phage S15 hybridized to the oligonucleotide S15). In addition, under the experimental conditions used, the oligonucleotides essentially did not hybridize to the negative control phage template M13mp18. Two oligonucleotides were synthesized to the phage M13mp18: (1) a positive oligonucleotide which hybridizes to a conserved sequence in both M13mp18 and each of the GIT receptor or GIT binding site selected phages [designated M13 (positive)]; and (2) a negative oligonucleotide which only hybridizes to a sequence unique to the multiple cloning site of phage M13mp18 and which does not hybridize to any of the GIT receptor or GIT binding site selected phages.

In the case of the hSI pool of phages, only four phages were transported from the closed loop model into the portal circulation: phages S15, SNI-10, SNI-34 and SNI-38. The other phages, S21, S22, SNI-28, SNI-45, SNIAX-2, SNIAX-6 and SNIAX-8, were not transported from the GIT into the portal circulation.

5 In addition, phages SNI-10 and to a lesser extent phages S15 and S22 were found in the intestine samples or fractions, whereas the other phages were not. There was a very low presence (<0.1%) of the phage M13mp18 in the Int-G samples. These results show that phages can be further selected from pre-selected libraries, permitting the identification of phages which are transported
10 from the GIT closed loop into the portal circulation or phages which bind to or are internalized by intestinal tissue.

In the case of the D2H pool of phages, there was a rank order by which phages were transported from the GIT closed loop model into the portal circulation, with phages DCX11 and DAB10 preferably transported, followed by phages DCX8, DAB30, DAB3 and
15 DAB7. A number of phages from this pool were not transported into the portal circulation, including phages DAB18, DAB24, DAX15, DAX24, DAX27, DCX26, DCX36, DCX39, DCX42, DCX45. There is a very low level of transport of phage DAX23 from the GIT into the portal circulation. Similarly, only some of the phages were found in the intestinal samples fractions, including phages
20 DAB30, DCX33, DAB7, DCX11, DCX45 and to a much lesser extent phages DAB3, DAB10, DCX8, DCX39, DCX42. Some phages were not found in the intestinal samples, including phages DAB18, DAB24, DAX15, DAX24, DCX26, and DCX36. There was a very low presence (<0.1%) of the phage M13mp18 in the Int-G samples. These results showed that phages can be further selected from pre-selected
25 libraries, permitting the identification of phages which are transported from the GIT closed loop into the portal circulation or phages which bind to or are internalized by intestinal tissue.

In the case of the HPT1 pool of phages, there was a rank order by which phages were transported from the GIT closed loop model into the portal or systemic circulation. Phage PAX2 (which
30 was used at a 4X concentration relative to the other phages in this pool) followed by phage HAX42 was found in the portal and

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systemic circulation; phage H40 was found in the systemic circulation only. None of the phages in this pool were found in the intestine samples or fractions. Phage M13mp18 was not found in the intestine fractions or systemic circulation, with very low incidence ($<0.001\%$) in the portal circulation. These results

5 show that phages can be further selected from pre-selected libraries, permitting the identification of phages which are transported from the GIT closed loop into the portal and/or systemic circulation or phages which bind to or are internalized by intestinal tissue.

In the case of the hPEPT1 pool of phages, the phages PAX2

10 and H40 were also included in this pool. A number of phages from this pool were found in the portal circulation, including phages P31 (SEQ ID NO:43), PAX46, PAX9, H40, PAX17, PAX40, PAX2, PAX14, 5PAX3 and 5PAX12. A number of phages were not found in the portal blood including the negative control phage M13mp18, PAX15, PAX16, PAX18, PAX35, PAX38, PAX43, PAX45, P90, 5PAX5 and 5PAX7.

15 The only phage found in the systemic circulation were phages 5PAX5 and P31 (SEQ ID NO:43). In addition, there was preferential binding of some phages to the intestine, including phages 5PAX12, 5PAX7, 5PAX3, H40, P31 (SEQ ID NO:43), PAX9, and to a lesser extent phages PAX38 and PAX15. Some phages were not found in the intestine samples, including the negative control

20 phage M13mp18 and the phages PAX2, PAX14, PAX16, PAX18, PAX35, PAX45, PAX46, P90 and 5PAX5. These results show that phages can be further selected from pre-selected libraries, permitting the identification of phages which are transported from the GIT closed loop into the portal and/or systemic circulation or phages which bind to or are internalized by intestinal tissue.

25

Further Characterization of Select Sequences

Following initial screening of the four recombinant receptor sites (hPEPT1, HPT1, D2H, hSI) of the gastrointestinal tissue, with the phage display libraries, a series of phage were isolated which showed preferential binding to the respective

30 target receptor sites in comparison to negative control protein

BSA protein and the recombinant protein recombinant human tissue factor (hTF) (which, like the recombinant receptors of the gastrointestinal tissue, contained a poly-histidine tag at its NH₂-terminal end). In subsequent experiments same titers of the selected phage which bound to each target receptor site were
5 combined into a single pool (i.e., one pool of HPT1 binding phage, one pool of hPEPT1 binding phage, one pool of D2H binding phage, and one pool of hSI binding phage). Each pool was supplemented with an equivalent titer of the negative control phage M13mp18. These phage pools were injected into a closed duodenal loop region of rat intestinal tissue and subsequently
10 phage was harvested and recovered which was bound to and retained by the intestinal tissue and/or was absorbed from the intestinal loop into the portal and/or systemic circulation. In addition, a selection of the initial phages which bound to the target recombinant receptor site were analyzed for binding to either fixed Caco-2 cells and/or to fixed C2BBel cells. The selection
15 of the final lead peptide sequences was based on the ability of the phage, coding for that peptide sequence (1) to bind to the target recombinant receptor site *in vitro* in preference to its binding to the negative control proteins BSA and/or hTFs, (2) to bind to rat intestinal tissue following injection into a closed duodenal loop of rat intestinal tissue in preference to the
20 negative control phage M13mp18, (3) to be absorbed from rat intestinal tissue into either the portal and/or systemic circulation following injection into a closed duodenal loop of rat intestinal tissue in preference to the negative control phage M13mp18, and (4) to bind to either fixed Caco-2 cells or fixed
25 C2BBel cells in phage binding studies in preference to the negative control phage M13mp18. Peptides were also selected with consideration to the ease of chemical synthesis.

6.9. GST Fusion Proteins of GIT Targeting Peptides **Construction of GST Fusion Proteins of GI** **Targeting Peptides**

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Glutathione S-transferase (GST) vectors encoding fusion proteins of GI targeting peptides were constructed in the vector pGEX4T-2 (source, Pharmacia Biotech, Piscataway, NJ). Briefly, single-strand DNA from the clones of interest were amplified by the polymerase chain reaction. The amplified DNA was then
5 cleaved with the restriction enzymes XhoI and NotI and then ligated into SalI/NotI cleaved pGEX4T-2. Following transformation, the DNA sequence for each construct was verified by sequencing.

For construction of the truncated versions of the GST fusion proteins, where the inserted sequence was less than 45
10 base pairs, overlapping oligonucleotides containing cohesive SalI and NotI termini, and encoding the sequence of interest, were annealed and then ligated directly into SalI/NotI cleaved pGEX4T-2. Following transformation, the DNA sequence for each construct was verified.

A diagrammatic representation of the various GST fusion
15 protein constructs that have been synthesized is indicated in Figures 5A-5C.

Expression and Purification of GST Fusion Proteins

Escherichia coli BL21 cells containing GST fusion
20 protein constructs were grown overnight in 2X YT media containing 100 µg/ml ampicillin (2X YT/amp). Overnight cultures were diluted 1:100 in 2X YT broth (100 ml), and cells were grown to an A₆₀₀ of 0.5 at 30°C, induced with 1mM isopropyl-1-thio-B-D-galactopyranoside, and grown for an additional 3 h. Cells were harvested by centrifugation and
25 resuspended in 5 ml of PBS containing a mixture of the proteinase inhibitors (Boehringer/Mannheim). Cells were sonicated on ice, and the cell lysates were centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatant fractions were reacted for 30 minutes at room temperature with 2 ml of a 50% slurry of glutathione-Sepharose® 4B, washed 3 times with 1.5 ml of PBS (at
30 room temperature), and the bound GST fusion proteins were eluted by reaction for 10 minutes at room temperature with 3 X 1ml of 10

mM reduced glutathione in 50 mM Tris HCl pH 8.0. Protein was quantified by the Bio-Rad protein assay followed by characterization by SDS-polyacrylamide gel electrophoresis.

ELISA of GST fusion peptides

5 The standard ELISA procedure was modified as follows. GST proteins were diluted to an appropriate concentration in PBS containing 1%BSA and 0.05% Tween20 (1%BPT), titered and incubated one hour at room temperature. Following five washes an anti-GST monoclonal antibody was added (Sigma, St. Louis Clone GST-2
10 more washes goat anti-mouse IgG2b-HRP was added (Southern Biotechnology Associates Inc., Birmingham, AL, diluted 1:4000 in 1%BPT) and incubated one hour. After five washes plates were developed with TMB peroxidase substrate (Kirkegard and Perry, Gaithersburg, MD). All data is presented with background binding subtracted.

15 Figure 6 shows the binding of GST-SNi10, GST-SNi34 and GST alone to the hSI receptor and to fixed C2BBel cells.

GST Fusion Proteins of Selected GIT Targeting Peptides

Results show that GST-DXB8, GST-PAX2, GST-P31,
20 GST-SNi10 and GST-SNi34 bound fixed Caco-2 or C2BBel cells (Figures 7 and 8) relative to GST control binding. GST-HAX42, GST-5PAX5, all showed weak to moderate binding relative to GST control.

Interestingly, P31 truncation 103-GST (SEQ ID NO: 185) fusion protein bound almost as well as full-length P31 (SEQ ID
25 NO:43) to fixed Caco-2 cells (A). This suggests the portion of the P31 sequence (SEQ ID NO:43) responsible for binding resides in this portion. PAX2.107 bound similarly to full-length PAX2; therefore, this portion most likely contains the amino acid sequence responsible for binding (B). In preliminary assays, none of the DCX8 truncations bound similarly to full-length DCX8
30 to Caco-2 cells suggesting the binding region spans more than one of these pieces.

Inhibition of Binding by Synthetic Peptides

Binding of GST-P31 to fixed C2BBel Cells

The standard ELISA procedure was modified as follows. GST fusion proteins and peptides were diluted to an appropriate concentration in PBS containing 1% BSA and 0.05% Tween 20. Peptides were titrated, a constant concentration of diluted GST protein was added to titrated peptides and the mixture was incubated one hour at room temperature. Following five washes, an anti-GST monoclonal antibody was added (Sigma, St. Louis Clone GST-2 diluted 1:10,000 in 1% BPT) and incubated one hour. After five more washes goat anti-mouse IgG2b-HRP was added (Southern Biotechnology Associates Inc., Birmingham, AL, diluted 1:4000 in 1% BPT) and incubated one hour. After five washes plates were developed with TMB peroxidase substrate (Kirkegard and Perry, Gaithersburg, MD). All data is presented with background binding subtracted.

Figures 9A and 9B show the inhibition of GST-P31 binding to C2BBel fixed cells. The peptide competitors are ZElan024 (SEQ ID NO:288) which is the dansylated peptide version of P31 (SEQ ID NO:43) and ZElan044 (SEQ ID NO:310), ZElan049 (SEQ ID NO:315) and ZElan050 (SEQ ID NO:316) which are truncated, dansylated pieces of P31 (SEQ ID NO:43). Data is presented as O.D. vs. peptide concentration and as percent inhibition of GST-P31 binding vs. peptide concentration. Uncompeted GST-P31 binding was considered as 100% binding. IC_{50} values are estimates using the 50% line on the percent inhibition graph.

GST-P31 and GST-PAX2 exhibited no crossreactive binding to ZElan024 (P31) (SEQ ID NO: 286) and ZElan018 (PAX2) (SEQ ID NO:281) at the 0.5 μ g/ml concentration used in competition assays. GST-HAX42 exhibited crossreactivity to ZElan018 (PAX2) (SEQ ID NO:281) and ZElan021 (HAX42) (SEQ ID NO:281) at the 5 μ g/ml concentration used in competition assays.

Figures 10A-10C present a compilation of data generated by competition ELISA of GST-P31, GST-PAX2, GST-SNi10 and GST-HAX42 versus various dansylated peptides on fixed C2BBel cells. IC_{50} values are in μ M and include ranges determined from

multiple assays. The GST/C2BBe1 column is a summary of GST protein binding to fixed C2BBe1 cells.

Binding to fixed Caco-2 Cells

5 Caco-2 cells were fixed, treated with phenylhydrazine and blocked as described above. Synthetic peptides (100µg/ml) were applied in duplicate to Caco-2 cells and serially diluted down the 96-well plate. The corresponding GST-peptide fusion protein (10µg) was added to each well and the plates were
10 incubated for 2h at room temperature with agitation. Binding of the GST-peptide fusion proteins to the cells was assayed using the ELISA technique described above. GST-P31 binding was inhibited by ZElan024 (SEQ ID NO:288), ZElan028 (SEQ ID NO:294) and ZElan031 (SEQ ID NO:297) as well as the two D forms ZElan053 (SEQ ID NO:319) and ZElan054 (SEQ ID NO:320). GST-PAX2 binding
15 was inhibited by ZElan032 (SEQ ID NO:298), ZElan033 (SEQ ID NO:299), and ZElan035 (SEQ ID NO:301). GST-HAX42 binding was not inhibited by ZElan021 (SEQ ID NO:285) (full length HAX42) but it was inhibited by ZElan018 (SEQ ID NO:281) (PAX2) and ZElan026
20 (SEQ ID NO:290) and ZElan038 (SEQ ID NO:304) (scrambled PAX2 peptides).

Transport and Uptake of GST-Peptide Fusions into Live Caco-2 Cells

25 Transport and uptake of GST-peptide fusions and deletion derivatives across cultured polarized Caco-2 monolayers over 4 hours in HBSS buffer was examined using an anti-GST ELISA assay. In another experiment, transport and uptake of GST-peptide fusions and deletion derivatives across cultured
30

polarized Caco-2 monolayers over 24 hours in serum-free medium (SFM) was examined using an anti-GST ELISA assay.

Materials

5 Buffered Hank's balanced salt solution (bHBSS) = 1x HBSS (Gibco CN.14065-031) supplemented with 0.011M glucose (1g/l), 25 mM Hepes (15 mM acid (3.575g/l; Sigma CN.H3375); 10mM base (2.603g/l; Sigma CN.H1016)].

 Chloroquine: Made up as 10mM solution in water [Sigma
10 CN C6628]

 Lysate buffer: 30 mM Tris-HCl pH8.0; 1mM EDTA

 Serum-free medium (SFM) is normal medium without serum.

Method

15 a) 4h HBSS study: Transepithelial electrical flux (TER) across the Caco-2 monolayers grown on snapwells (passage 33; 23 days old) was measured to confirm monolayer integrity before beginning the experiment. The medium was removed and the cells were washed once with bHBSS. bHBSS containing 100µM
20 chloroquine was added and the cells were incubated for 2h at 37°C. The bHBSS+chloroquine was replaced with 0.5ml bHBSS containing GST-peptide fusions (100µg/ml) and the cells were incubated as before. Basolateral samples were removed at the following times: 0, 0.5h, 2h, and 4h. At 4h, TER was measured,
25 the apical medium was sampled and the apical reservoir was washed 6 times with HBSS. The cells were allowed to lyse for 1h on ice in lysate buffer, after which, lysate sample was collected. All samples were stored at -70°C until assay by anti-GST ELISA. Before analysis, samples were normalized for protein content
30 relative to each other using a BioRad protein assay.

b) 24h SFM study: Transepithelial electrical flux (TER) across the Caco-2 monolayers grown on snapwells (passage 33; 23 days old) was measured to confirm monolayer integrity before beginning the experiment. The medium was removed and the cells were washed once with SFM. SFM containing GST-peptide fusions (100µg/ml) was added to the cells which were incubated at 37°C for 24h at 5% CO₂. After 24 hours, TER readings were taken, and samples from the basolateral and apical reservoirs were removed. The apical reservoir was washed 6 times with PBS. The cells were allowed to lyse for 1h on ice in lysate buffer, after which lysate sample was collected. All samples were stored at -70° until assay by anti-GST ELISA. Before analysis, samples were normalized for protein content relative to each other using a BioRad protein assay.

15

Results

All of the GST-peptide fusions and controls examined were transported across live Caco-2 monolayers. Full-length GST-P31 and GST-DCX8, but not truncations of these molecules had a higher flux than GST alone.

Internalization of GST-peptide fusions into polarized Caco-2 cells was investigated in two experiments. In experiment 1, 15µg of GST-peptide fusion was applied in bHBSS and internalized GST-peptide was recovered by lysing the cells after 4h. In experiment 2, 10µg of GST-peptide was applied in either a) bHBSS (lysate recovered after 4h), or b) serum-free medium (lysate recovered after 24h).

Figure 11A describes complete transport of GST-peptide across a polarized Caco-2 monolayer and does not necessarily refer to internalization, i.e., the GST-peptide was recovered

from the basolateral reservoir of a snapwell but the proteins could have crossed the barrier by the paracellular route.

Effect of Thrombin Cleavage on Binding of GST-Peptide Fusions to Fixed Caco-2 Cells

5

Binding of intact and thrombin-cleaved GST-peptide fusions to fixed Caco-2 cells was compared. Reduced binding of the thrombin-cleaved GST-peptide fusions relative to intact fusions indicates that the peptide component of the fusion, and not the GST domain, mediates binding.

Method

Confluent Caco-2 monolayers grown in 96-well plates (p38) were fixed and treated with 0.1% phenylhydrazine before blocking with 0.1% BSA in PBS. Thirty micrograms of each GST-peptide was treated with bovine thrombin (1 μ /ml; 0.4 NIH units; Sigma CN.T9681) for 18h at room temperature in 20mM Tris-HCl pH8.0, 150mM NaCl, 2.5mM CaCl₂. Controls were similarly treated without addition of thrombin. Ten micrograms of each GST-peptide fusion was removed for PAGE analysis, and 10 μ g of fusions were added in duplicate to the fixed Caco-2 cells before 5-fold serial dilutions (1% BPT diluent). The fusions were allowed to bind for 1h at room temperature. Following 6 washes with 1% BPT, binding was assayed by ELISA.

25

Results

Results are shown in Figure 12.

Conclusions:

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PAGE analysis confirmed that the GST-peptide fusions were effectively cleaved with thrombin. Cleavage with thrombin significantly reduced detection of binding of GST-P31.103 (SEQ ID NO: 183), GST-PAX2.106 (SEQ ID NO: 188), GST-DCX8, GST-SNi10 to 5 fixed Caco-2 cells, indicating that the peptide component, and not the GST domain, mediates binding.

6.10. Synthesis of Peptides

6.10.1. Procedure For Solid Phase Synthesis

10 Peptides may be prepared by methods that are known in the art. For example, in brief, solid phase peptide synthesis consists of coupling the carboxyl group of the C-terminal amino acid to a resin and successively adding N-alpha protected amino acids. The protecting groups may be any known in the art.
15 Before each new amino acid is added to the growing chain, the protecting group of the previous amino acid added to the chain is removed. The coupling of amino acids to appropriate resins is described by Rivier et al., U.S. Patent No. 4,244,946. Such solid phase syntheses have been described, for example, by
20 Merrifield, 1964, J. Am. Chem. Soc. 85:2149; Vale et al., 1981, Science 213:1394-1397; Marki et al., 1981, J. Am. Chem. Soc. 103:3178 and in U.S. Patent Nos. 4,305,872 and 4,316,891. In a preferred aspect, an automated peptide synthesizer is employed.

By way of example but not limitation, peptides can be
25 synthesized on an Applied Biosystems Inc. ("ABI") model 431A automated peptide synthesizer using the "Fastmoc" synthesis protocol supplied by ABI, which uses
2-(1H-Benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium
hexafluorophosphate ("HBTU") (R. Knorr et al., 1989, Tet. Lett.,
30 30:1927) as coupling agent. Syntheses can be carried out on 0.25

mmol of commercially available

4-(2',4'-dimethoxyphenyl-(9-fluorenyl-methoxycarbonyl)-aminomethyl)-phenoxy polystyrene resin ("Rink resin" from Advanced ChemTech) (H. Rink, 1987, Tet. Lett. 28:3787). Fmoc amino acids (1 mmol) are coupled according to the Fastmoc protocol. The following side chain protected Fmoc amino acid derivatives are used: FmocArg(Pmc)OH; FmocAsn(Mbh)OH; FmocAsp(^tBu)OH; FmocCys(Acm)OH; FmocGlu(^tBu)OH; FmocGln(Mbh)OH; FmocHis(Tr)OH; FmocLys(Boc)OH; FmocSer(^tBu)OH; FmocThr(^tBu)OH; FmocTyr(^tBu)OH. [Abbreviations: Acm, acetamidomethyl; Boc, tert-butoxycarbonyl; ^tBu, tert-butyl; Fmoc, 9-fluorenylmethoxycarbonyl; Mbh, 4,4'-dimethoxybenzhydryl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Tr, trityl].

Synthesis is carried out using N-methylpyrrolidone (NMP) as solvent, with HBTU dissolved in N,N-dimethylformamide (DMF). Deprotection of the Fmoc group is effected using approximately 20% piperidine in NMP. At the end of each synthesis the amount of peptide present is assayed by ultraviolet spectroscopy. A sample of dry peptide resin (about 3-10 mg) is weighed, then 20% piperidine in DMA (10 ml) is added. After 30 min sonication, the UV (ultraviolet) absorbance of the dibenzofulvene-piperidine adduct (formed by cleavage of the N-terminal Fmoc group) is recorded at 301 nm. Peptide substitution (in mmol g⁻¹) can be calculated according to the equation:

$$\text{substitution} = \frac{A \times v}{7800 \times w} \times 1000$$

where A is the absorbance at 301 nm, v is the volume of 20% piperidine in DMA (in ml), 7800 is the extinction coefficient (in

6.10.4. Conjugation of Peptides to Other Molecules

The peptides of the present invention may be linked to other molecules (e.g., a detectable label, a molecule facilitating adsorption to a solid substratum, or a toxin, according to various embodiments of the invention) by methods that are well known in the art. Such methods include the use of homobifunctional and heterobifunctional cross-linking molecules.

The homobifunctional molecules have at least two reactive functional groups, which are the same. The reactive functional groups on a homobifunctional molecule include, for example, aldehyde groups and active ester groups. Homobifunctional molecules having aldehyde groups include, for example, glutaraldehyde and subaraldehyde. The use of glutaraldehyde as a cross-linking agent was disclosed by Poznansky et al., 1984, Science 223:1304-1306.

Homobifunctional molecules having at least two active ester units include esters of dicarboxylic acids and N-hydroxysuccinimide. Some examples of such N-succinimidyl esters include disuccinimidyl suberate and dithio-bis-(succinimidyl propionate), and their soluble bis-sulfonic acid and bis-sulfonate salts such as their sodium and potassium salts. These homobifunctional reagents are available from Pierce, Rockford, Illinois.

The heterobifunctional molecules have at least two different reactive groups. Some examples of heterobifunctional reagents containing reactive disulfide bonds include N-succinimidyl 3-(2-pyridyl-dithio)propionate (Carlsson et al., 1978, Biochem J. 173:723-737), sodium S-4-succinimidylloxycarbonyl-alpha-methylbenzylthiosulfate, and

4-succinimidylloxycarbonyl-alpha-methyl-(2-pyridyldithio)toluene.
N-succinimidyl 3-(2-pyridyldithio)propionate is preferred. Some
examples of heterobifunctional reagents comprising reactive
groups having a double bond that reacts with a thiol group
5 include succinimidyl
4-(N-maleimidomethyl)cyclohexane-1-carboxylate and succinimidyl
m-maleimidobenzoate.

Other heterobifunctional molecules include succinimidyl
3-(maleimido)propionate, sulfosuccinimidyl
10 4-(p-maleimido-phenyl)butyrate, sulfosuccinimidyl
4-(N-maleimidomethyl-cyclohexane)-1-carboxylate,
maleimidobenzoyl-N-hydroxy-succinimide ester. The sodium
sulfonate salt of succinimidyl m-maleimidobenzoate is preferred.
Many of the above-mentioned heterobifunctional reagents and their
15 sulfonate salts are available from Pierce.

Additional information regarding how to make and use
these as well as other polyfunctional reagents may be obtained
from the following publications or others available in the art:
Carlsson et al., 1978, Biochem. J. 173:723-737; Cumber et al.,
20 1985, Methods in Enzymology 112:207-224; Jue et al., 1978,
Biochem 17:5399-5405; Sun et al., 1974, Biochem. 13:2334-2340;
Blattler et al., 1985, Biochem. 24:1517-152; Liu et al., 1979,
Biochem. 18:690-697; Youle and Neville, 1980, Proc. Natl. Acad.
Sci. USA 77:5483-5486; Lerner et al., 1981, Proc. Natl. Acad.
25 Sci. USA 78:3403-3407; Jung and Moroi, 1983, Biochem. Biophys.
Acta 761:162; Caulfield et al., 1984, Biochem. 81:7772-7776;
Staros, 1982, Biochem. 21:3950-3955; Yoshitake et al., 1979, Eur.
J. Biochem. 101:395-399; Yoshitake et al., 1982, J. Biochem.
92:1413-1424; Pilch and Czech, 1979, J. Biol. Chem.
30 254:3375-3381; Novick et al., 1987, J. Biol. Chem. 262:8483-8487;

Lomant and Fairbanks, 1976, J. Mol. Biol. 104:243-261; Hamada and Tsuruo, 1987, Anal. Biochem. 160:483-488; Hashida et al., 1984, J. Applied Biochem. 6:56-63.

Additionally, methods of cross-linking are reviewed by Means and Feeney, 1990, Bioconjugate Chem. 1:2-12.

6.10.4.1. Biotinylation of Peptides

Methods of biotinylating peptides are well known in the art. Any convenient method may be employed in the practice of the invention. For example, the following procedure was used. Ten micrograms of peptide was dissolved in 100 μ l of 0.1 % acetic acid. PBS (900 μ l) and 3.3 mg of biotin-LC-NHS (Pierce, Rockford, IL) was added. Following incubation for 30 minutes at room temperature the biotinylated peptides were purified over a Superose 12 column (Pharmacia, Piscataway, NJ).

6.10.5. Synthetic Peptides

Tables 19, 20 and 21 provide the primary structure for various synthetic peptides manufactured in the practice of the present invention.

| Table 19 | | |
|------------------|------------------|---|
| SEQ ID NO. | Peptide name | Sequence |
| 266 | ELAN005 | H ₂ N-C-K(dns) - FITKALGISYGRKKRRQRRRPPQGSQTHQVS LSKQ-CONH ₂ |
| 267 | ELAN006 | Ac-CLNGGVKMYVESVDRYVC-CONH ₂ |
| 268 | FITC-ELAN 006 | Ac-CLNGGVK(FITC)MYVESVDRYVC-CONH ₂ |

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|----|-----|------------------|--|
| 5 | 269 | ELAN006ii | H ₂ N-C-K(dns)-RLNGGVSMYVESVDRYVCR-CONH ₂ |
| | 167 | ELAN007 | H ₂ N-RIAGLPWYRCRTVAFETGMQNTQLCSTIVQLSFTPEE-COOH |
| | 193 | ELAN007ii | H ₂ N-KKRIAGLPWYRCRTVAFETGMQNTQLCSTIVQLSFTPEE-CONH ₂ |
| | 270 | bZElan008 (P31) | biotin-K(dns) SARDSGPAEDGSRAVRLNGVENANTRKSSR SNPRGRRHP-COOH |
| | 271 | bZElan009 | biotin-K(dns) SSADAEKCAGSLLWWGRQNNSGCGSPTKKH LKHRNRSQTSSSSHG-COOH |
| 10 | 168 | ELAN010 | H ₂ N-REFAERRLWGCDDLSWRLDAEGCGPTPSNRAVKHRKPRPR SPAL-COOH |
| | 272 | bZElan010 | biotin-K(dns) REFAERRLWGCDDLSWRLDAEGCGPTPSNR AVKHRKPRPRSPAL-COOH |
| | 169 | ELAN012 | H ₂ N-SGSHSGGMNRAYGDVFRELDRWYATSHHTRPTPQLPRGP N-COOH |
| | 273 | bELAN012 | biotin-SGSHSGGMNRAYGDVFRELDRWYATSHHTRPTPQL PRGP N-COOH |
| | 274 | ZElan012 | H ₂ N-K(dns) SGSHSGGMNRAYGDVFRELDRWYATSHHTRPTP QLPRGP N-COOH |
| 15 | 249 | ELAN013 | H ₂ N-SGSPPCGGSWGRFMQGGLFGGRTDGC GAHRNRTSASLEPP SSDY-CONH ₂ |
| | 250 | ELAN014 | H ₂ N-SHSGGMNRAYGDVFRELDRWNATSHHTRPTPQLPRGPNS -CONH ₂ |
| | 275 | bZElan014 | biotin-K(dns) SHSGGMNRAYGDVFRELDRWNATSHHTRP TPQLPRGPNS-CONH ₂ |
| | 276 | ZElan014 | H ₂ N-K(dns) SHSGGMNRAYGDVFRELDRWNATSHHTRPTPQL PRGPNS-CONH ₂ |
| | 277 | ZElan015 (DCX11) | H ₂ N-K(dns) SQGSKQCMQYRTGRLTVGSEYCGMNP ARHATPA YPARLLPRYR-CONH ₂ |
| 20 | 278 | ZElan016 (SNI10) | H ₂ N-K(dns) RVGQCTDSDVRRPWARS CAHQCGAGTRNSHGCI TRPLRQASAH-CONH ₂ |
| | 279 | bZElan017 | biotin-K(dns) SGSGRVGQCTDSDVRRPWARS CA-CONH ₂ |
| | 280 | ZElan017 | H ₂ N-K(dns) RVGQCTDSDVRRPWARS CA-CONH ₂ |
| | | ZElan018 (PAX2) | H ₂ N-K(dns) STPPSREAYSRPYSVDS DSDTNAKHSSHNRLRT RSRPNG-CONH ₂ |
| | 282 | ZElan019 (5PAX5) | H ₂ N-K(dns) RGSTGTAGGERSGVLNLHTRDNASGSGFKPWYPS NRGHK-CONH ₂ |
| 25 | 283 | ZElan020 (CY09) | H ₂ N-K(dns) SGSGLYANPGMYSLHSPA-CONH ₂ |
| | 284 | bZElan020 (CY09) | biotin-K(dns) SGSGLYANPGMYSLHSPA-CONH ₂ |
| | 285 | ZElan021 (HAX42) | H ₂ N-K(dns) SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVF NRRRPSAIP T-CONH ₂ |
| | 286 | ZElan022 (SNI34) | H ₂ N-K(dns) SPCGGSWGRFMQGGLFGGRTDGC GAHRNRTSASL EPPSSDY-CONH ₂ |
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| 5 | 287 | ZElan023 (DCX8) | H ₂ N-K (dns) RYKHDIGCDAGVDKKSSSVRGGCGAHSSPPRAGR GPRGTMVSRL-CONH ₂ |
| | 288 | ZElan024 (P31) | H ₂ N-K (dns) SARDSGPAEDGSRAVRLNGVENANTRKSSRSNPR GRRHPGG-CONH ₂ |
| | 289 | ZElan025 (DAB10) | H ₂ N-K (dns) SKSGEGGDSSRGETGWARVRSHAMTAGRFRWYNQ LPSDR-CONH ₂ |
| | 290 | ZElan026 (PAX2/con trol) | H ₂ N-K (dns) SEANLDGRKSRYSSPRRNSSTRPRTSPNSVHARY PSTDHD-CONH ₂ |
| | 291 | bELan027 (PAX2) | biotin-SGSGSTPPSREAYSRPYSVDSDDSDTNAKHSSHNRL RTRSRPNG-CONH ₂ |
| 10 | 251 | 18C21 | H ₂ N-DTNAKHSSHNRLRTRSRPNG-CONH ₂ |
| | 292 | Fmoc-Z16N 23 | Fmoc-K (dns) RVGQCTDSDVRRPWARSCAHQG-COOH |
| | 252 | 16C23 | H ₂ N-CGAGTRNSHGCITRPLRQASAHG-CONH ₂ |
| | 293 | Z16C23 | H ₂ N-K (dns) CGAGTRNSHGCITRPLRQASAHG-CONH ₂ |
| | 294 | ZElan028 (P31 fragment) | H ₂ N-K (dns) ENANTRKSSRSNPRGRRHPG-CONH ₂ |
| 15 | 295 | ZElan029 (P31 fragment) | H ₂ N-K (dns) TRKSSRSNPRG-CONH ₂ |
| | 296 | ZElan030 (P31 fragment) | H ₂ N-K (dns) ENANTRKSSRSNPRG-CONH ₂ |
| | 297 | ZElan031 (P31 fragment) | H ₂ N-K (dns) TRKSSRSNPRGRRHPG-CONH ₂ |
| | 298 | ZElan032 (PAX2 fragment) | H ₂ N-K (dns) TNAKHSSHNRLRTRSRPN-CONH ₂ |
| | 299 | ZElan033 (PAX2 fragment) | H ₂ N-K (dns) TNAKHSSHNRLRTR-CONH ₂ |
| 20 | 300 | ZElan034 (PAX2 fragment) | H ₂ N-K (dns) SSHNRRLRTRSRPN-CONH ₂ |
| | 301 | ZElan035 (PAX2 fragment) | H ₂ N-K (dns) SSHNRRLRTR-CONH ₂ |
| | 302 | ZElan036 (SNI10 fragment) | H ₂ N-K (dns) VRRPWARSCAHQCGAGTRNS-CONH ₂ |

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| | 303 | ZElan037 (SNI10 fragment) | H ₂ N-K (dns) CTDSDVRRPWARSC-CONH ₂ |
| | 304 | ZElan038 (PAX2/con trol) | H ₂ N-K (dns) SRANTDGRKSRYSPPRRNSSTEPRLSPNSVHARY PSTDHD-CONH ₂ |
| 5 | 1923 05 | ZElan039 (P31 fragment) | H ₂ N-K (dns) ENANTRKSSR-CONH ₂ |
| | 306 | ZElan040 (P31 fragment) | H ₂ N-K (dns) SNPRGRRHPG-CONH ₂ |
| 10 | 307 | ZElan041 (P31 fragment) | H ₂ N-K (dns) ENANT-CONH ₂ |
| | 308 | ZElan042 (P31 fragment) | H ₂ N-K (dns) ANTRKS-CONH ₂ |
| | 309 | ZElan043 (P31 fragment) | H ₂ N-K (dns) TRKSS-CONH ₂ |
| 15 | 310 | ZElan044 (P31 fragment) | H ₂ N-K (dns) RKSSR-CONH ₂ |
| | 311 | ZElan045 (P31 fragment) | H ₂ N-K (dns) KSSRSN-CONH ₂ |
| 20 | 312 | ZElan046 (P31 fragment) | H ₂ N-K (dns) SSRSNPG-CONH ₂ |
| | 313 | ZElan047 (P31 fragment) | H ₂ N-K (dns) RSNPRG-CONH ₂ |
| | 314 | ZElan048 (P31 fragment) | H ₂ N-K (dns) SNPRG-CONH ₂ |
| 25 | 315 | ZElan049 (P31 fragment) | H ₂ N-K (dns) PRGRRH-CONH ₂ |
| | 316 | ZElan050 (P31 fragment) | H ₂ N-K (dns) RRHPG-CONH ₂ |
| 30 | 317 | ZElan051 (HepC) | H ₂ N-K (dns) KSSRGN-CONH ₂ |

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|----|-----|---------------------------------|---|
| 5 | 318 | ZElan052 (HepC) | H ₂ N-K (dns) KTSERSQPRGRRQPG-CONH ₂ |
| | 319 | ZElan053 (P31 analog) | H ₂ N-K (dns) TrKSSrSNPrGrrHPG-CONH ₂ |
| | 320 | ZElan054 (P31 analog) | H ₂ N-K (dns) TRKSSrSNPRGrRHPG-CONH ₂ |
| | 321 | ZElan055 (PAX2 fragment) | H ₂ N-K (dns) TNAKHSSHN-CONH ₂ |
| | 322 | ZElan056 (PAX2 fragment) | H ₂ N-K (dns) RRLRTRSRPN-CONH ₂ |
| 10 | 323 | ZElan057 (PAX2 fragment) | H ₂ N-K (dns) RRLRTRSR-CONH ₂ |
| | 324 | ZElan058 (PAX2 fragment) | H ₂ N-K (dns) RRLRTR-CONH ₂ |
| | 325 | ZElan059 (PAX2 analog) | H ₂ N-K (dns) rrLrTrSrPN-CONH ₂ |
| 15 | 326 | ZElan060 (HAX42 fragment) | H ₂ N-K (dns) SDHALGTNLRSDNAKEPGDYNCCGNG-CONH ₂ |
| | 327 | ZElan061 (HAX42 fragment) | H ₂ N-K (dns) GDYNCCGNGNSTGRKVFNRRRPSAIP-CONH ₂ |
| | 328 | ZElan062 (HAX42 fragment) | H ₂ N-K (dns) SDHALGTNLRSDNAKEPG-CONH ₂ |
| | 329 | ZElan063 (HAX42 fragment) | H ₂ N-K (dns) GDYNCCGNGNSTG-CONH ₂ |
| | 330 | ZElan064 (HAX42 fragment) | H ₂ N-K (dns) RKVFNRRRPSAIP-CONH ₂ |
| 25 | 331 | ZElan065 (HAX42 fragment) | H ₂ N-K (dns) RKVFNRRRPS-CONH ₂ |
| | 332 | ZElan066 (HAX42 fragment) | H ₂ N-K (dns) NRRRPSAIP-CONH ₂ |
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| 5 | 333 | ZElan067 (HAX42 fragment) | H ₂ N-K (dns) NRRRPS-CONH ₂ |
| | 55 | Elan018 | H ₂ N-STPPSREAYSRPYSVDSDDSDTNAKHSSHNRRLRTRSRPNG |
| | 334 | (PAX2 no dns) | -CONH ₂ |
| | 52 | Elan021 | H ₂ N-SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVFNRRRPS |
| | 335 | (HAX42 no dns) | AIPT-CONH ₂ |
| 10 | 336 | ZElan070 (HAX42 fragment) | H ₂ N-K (dns) SDHALGTNLRSDNAKEPGDYNCCGNGNST-CONH ₂ |
| | 337 | ZElan071 (HAX42 fragment) | H ₂ N-K (dns) NLRSDNAKEPGDYNCCGNGNSTGRKVFNR-CONH ₂ |
| | 338 | ZElan072 (HAX42 fragment) | H ₂ N-K (dns) PGDYNCCGNGNSTGRKVFNRRPSAIPT-CONH ₂ |
| | 339 | ZElan073 (PAX2 fragment) | H ₂ N-K (dns) ASHNRRLRTR-CONH ₂ |
| | 340 | ZElan074 (PAX2 fragment) | H ₂ N-K (dns) SAHNRRLRTR-CONH ₂ |
| 20 | 341 | ZElan075 (PAX2 fragment) | H ₂ N-K (dns) SSANRRLRTR-CONH ₂ |
| | 342 | ZElan076 (PAX2 fragment) | H ₂ N-K (dns) SSHARLRTR-CONH ₂ |
| | 343 | ZElan077 (PAX2 fragment) | H ₂ N-K (dns) SSHNARLRTR-CONH ₂ |
| | 344 | ZElan078 (PAX2 fragment) | H ₂ N-K (dns) SSHNRALRTR-CONH ₂ |
| | 345 | ZElan079 (PAX2 fragment) | H ₂ N-K (dns) SSHNRRARTR-CONH ₂ |
| 30 | 346 | ZElan080 (PAX2 fragment) | H ₂ N-K (dns) SSHNRRLATR-CONH ₂ |
| | 347 | ZElan081 (PAX2 fragment) | H ₂ N-K (dns) SSHNRRLRAR-CONH ₂ |

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|---|-----|--------------------------------|---|
| 5 | 348 | ZElan082 (PAX2 fragment) | H ₂ N-K (dns) SSHNRRLRTA-CONH ₂ |
| | 349 | Elan035 | H ₂ N-SSHNRRLRTR-CONH ₂ |
| | 350 | ZElan083 (PAX2/con trol) | H ₂ N-K (dns) GRNHDVVSSNTHKSYRSPRSASYPRLSNDRTDRT EPAPSS-CONH ₂ |
| | 351 | ZElan084 (PAX2/con trol) | H ₂ N-K (dns) RNTRNKTSRLSANPHRSHR-CONH ₂ |
| | 352 | Elan032Z (PAX2 fragment) | H ₂ N-TNAKHSSHNRRLRTRSRPN K (dns) -CONH ₂ |
| | 353 | Elan057Z (PAX2 fragment) | H ₂ N-RRLRTRSRK (dns) -CONH ₂ |

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| TABLE 20 | | | |
| | Name | Description | Sequence |
| | | | SEQ. ID. No. |
| | ZElan087 | HAX42-1 (20 mer) | H ₂ N-K (dns) SDHALGTNLRSDNAKEPGDY 354 |
| | ZElan088 | HAX42-2 (20 mer) | H ₂ N-K (dns) SDNAKEPGDYNCCGNGNSTG 355 |
| | ZElan089 | HAX42-3 (15 mer) | H ₂ N-K (dns) SDHALGTNLRSDNAK 356 |
| 20 | ZElan090 | HAX42-4 (15 mer) | H ₂ N-K (dns) EPGDYNCCGNGNSTG 357 |
| | ZElan091 | HAX42-5 (14 mer) | H ₂ N-K (dns) PGDYNCCGNGNSTG 358 |
| | ZElan092 | HAX42-6 (10 mer) | H ₂ N-K (dns) PGDYNCCGNG 359 |
| | ZElan093 | HAX42-7 (10 mer) | H ₂ N-K (dns) NCCGNGNSTG 360 |
| | ZElan100 | P31 16 mer cyclic | H ₂ N-K (dns) Lys-TRKSSRSNPRGRRHPG 361 |
| 25 | | | |
| | ZElan101 | P31 16 mer cyclic D form | H ₂ N-K (dns) Lys-TrKSSrSNPrGrrHPG 362 |
| | | | |
| 30 | ZElan103 | PAX2 15 mer cyclic | H ₂ N-K (dns) Lys-TNAKHSSHNRRLRTR 363 |
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|----|---------------|-------------------------------------|--|-----|
| 5 | ZElan103 A | PAX2 15 mer cyclic (internal) | H ₂ N-K (dns) TNAKHSSCNRRRCRTR [] | 364 |
| | ZElan104 | PAX2 15 mer cyclic (internal) | H ₂ N-K (dns) TNAKHSSCNRRRLRCR [] | 365 |
| | ZElan105 | PAX2 Ala Scan 1 | H ₂ N-K (dns) ANAKHSSHNRRRLRTR | 366 |
| | ZElan106 | PAX2 Ala Scan 2 | H ₂ N-K (dns) TAAKNSSHNRRRLRTR | 367 |
| | ZElan107 | PAX2 Ala Scan 3 | H ₂ N-K (dns) TNGKNSSHNRRRLRTR | 368 |
| 10 | ZElan108 | PAX2 Ala Scan 4 | H ₂ N-K (dns) TNAAHSSHNRRRLRTR | 369 |
| | ZElan109 | PAX2 Ala Scan 5 | H ₂ N-K (dns) TNAKASSHNRRRLRTR | 370 |
| | ZElan110 | PAX2 Ala Scan 6 | H ₂ N-K (dns) TNAKHASHNRRRLRTR | 371 |
| | ZElan111 | PAX2 Ala Scan 7 | H ₂ N-K (dns) TNAKHSAHNRRRLRTR | 372 |
| | ZElan112 | PAX2 Ala Scan 8 | H ₂ N-K (dns) TNAKHSSANRRRLRTR | 373 |
| 15 | ZElan113 | PAX2 Ala Scan 9 | H ₂ N-K (dns) TNAKHSSHARRLRTR | 374 |
| | ZElan114 | PAX2 Ala Scan 10 | H ₂ N-K (dns) TNAKHSSHNARLRTR | 375 |
| | ZElan115 | PAX2 Ala Scan 11 | H ₂ N-K (dns) TNAKHSSHNARLRTR | 376 |
| | ZElan116 | PAX2 Ala Scan 12 | H ₂ N-K (dns) TNAKHSSHNRRARTR | 377 |
| | ZElan117 | PAX2 Ala Scan 13 | H ₂ N-K (dns) TNAKHSSHNRRLATR | 378 |
| 20 | ZElan118 | PAX2 Ala Scan 14 | H ₂ N-K (dns) TNAKHSSHNRRRLRAR | 379 |
| | ZElan119 | PAX2 Ala Scan 15 | H ₂ N-K (dns) TNAKHSSHNRRRLRTA | 380 |
| | ZElan123 | PAX2 15 mer cyclic D form | H ₂ N-K (dns) Lys-TNAKHSSHNrrLrTr | 381 |
| | ZElan124 | PAX2 15 mer D form | H ₂ N-K (dns) TNAKHSSHNrrLrTr | 382 |
| | ZElan125 | PAX2 10 mer cyclic | H ₂ N-K (dns) Lys-SSHNRRRLRTR [] | 383 |
| 25 | ZElan126 | PAX2 10 mer cyclic D form | H ₂ N-K (dns) Lys-SSHNrrLrTr [] | 384 |
| | ZElan127 | PAX2 10 mer cyclic | H ₂ N-K (dns) Lys-TNAKHSSHNr [] | 385 |
| | ZElan128 | PAX2 10 mer cyclic D form | H ₂ N-K (dns) Lys-TNAKHSSHNr [] | 386 |
| | ZElan129 | PAX2 15 mer | H ₂ N-K (dns) TNAKHSSHNRRRLRTR | 387 |
| | ZElan130 | HAX42 14 mer Ala Scan 1 | H ₂ N-K (dns) AGDYNCCGNGNSTG | 388 |
| 30 | | | | |

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|----|----------|-----------------------------|--|-----|
| | ZElan131 | HAX42 14 mer Ala Scan 2 | H ₂ N-K(dns) PADYNCCGNGNSTG | 389 |
| | ZElan132 | HAX42 14 mer Ala Scan 3 | H ₂ N-K(dns) PGAYNCCGNGNSTG | 390 |
| | ZElan133 | HAX42 14 mer Ala Scan 4 | H ₂ N-K(dns) PGDANCCGNGNSTG | 391 |
| 5 | ZElan134 | HAX42 14 mer Ala Scan 5 | H ₂ N-K(dns) PGDYACCGNGNSTG | 392 |
| | ZElan135 | HAX42 14 mer Ala Scan 6 | H ₂ N-K(dns) PGDYNACGNGNSTG | 393 |
| | ZElan136 | HAX42 14 mer Ala Scan 7 | H ₂ N-K(dns) PGDYNCAGNGNSTG | 394 |
| 10 | ZElan137 | HAX42 14 mer Ala Scan 8 | H ₂ N-K(dns) PGDYNCCANGNSTG | 395 |
| | ZElan138 | HAX42 14 mer Ala Scan 9 | H ₂ N-K(dns) PGDYNCCGAGNSTG | 396 |
| | ZElan139 | HAX42 14 mer Ala Scan 10 | H ₂ N-K(dns) PGDYNCCGNANSTG | 397 |
| | ZElan140 | HAX42 14 mer Ala Scan 11 | H ₂ N-K(dns) PGDYNCCGNGASTG | 398 |
| 15 | ZElan141 | HAX42 14 mer Ala Scan 12 | H ₂ N-K(dns) PGDYNCCGNGNATG | 399 |
| | ZElan142 | HAX42 14 mer Ala Scan 13 | H ₂ N-K(dns) PGDYNCCGNGNSAG | 400 |
| | ZElan143 | HAX42 14 mer Ala Scan 14 | H ₂ N-K(dns) PGDYNCCGNGNSTA | 401 |

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GST fusion proteins of GIT peptides are shown in Table

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Table 21

| Source | Clone # | GST Fusion Sequence | SEQ ID NO. |
|--------|---------|---|------------|
| DCX11 | 98 | gst-SQGSKQCMQYRTGRLTVGSEYCGMNPARGHATPAYPARLLPRYR | 213 |
| HAX42 | 99 | gst-SDHALGTNLRSDNAKEPGDYNC CGNGNSTGRKVFNNRRRPSA IPT | 214 |
| SNi34 | 100 | gst-SPCGGSGWGRFMQGLFGGRTDGC GAHRNRTSASLEPPSSDY | 215 |
| 5PAX5 | 97 | gst-RGSTGTAGGERSGVLNLHTRDNASGSGFKPWYPSNRG HK | 216 |
| SNi28 | 84 | gst-SHSGGMNRAYGDVFRELDRWNATSHHTRPTPQLPRGPN | 217 |
| SNi28 | 85 | gst-SHSGGMNRAY | 218 |
| SNi28 | 86 | gst-GDVFRELDR | 219 |
| SNi28 | 87 | gst-WNATSHHTRP | 220 |
| SNi28 | 88 | gst-TPQLPRGPN | 221 |
| SNi28 | 89 | gst-GDVFRELDRWNATSHHTRP | 222 |
| SNi28 | 90 | gst-WNATSHHTRPTPQLPRGPN | 223 |
| SNi28 | 91 | gst-GDVFRELDRWNATSHHTRPTPQLPRGPN | 224 |
| SNi28 | 92 | gst-SHSGGMNRAYGDVFRELDRWNATSAATRPTPQLPRGPN | 225 |
| P31 | 93 | gst-SARDSGPAEDGSRAVRLNGVENANTRKSSRSNPRGRRHP | 226 |
| P31 | 101 | gst-SARDSGPAEDGSRAVRLNG | 227 |
| P31 | 102 | gst-DGSRAVRLNGVENANTRKSSR | 228 |

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|-----|-----|-------------------------|-----|
| P31 | 103 | gst-ENANTRKSSRSNPRGRRHP | 229 |
| P31 | 110 | gst-ENANTRKSSR | 230 |

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| P31 | 111 | gst-RKSSRSNPRG | |
| P31 | 112 | gst-SNPRGRRHP | 232 |
| P31 | 119 | gst-TRKSSRSNPRG | 233 |
| PAX2 | 94 | gst-STPPSREAYSRPYSDSDTNAKHSSHNRRRLRTRSRPN | 234 |
| PAX2 | 104 | gst-STPPSREAYSRPYSDSDSD | 235 |
| PAX2 | 105 | gst-SRPYSDSDSDTNAKHSSHN | 236 |
| PAX2 | 106 | gst-TNAKHSSHNRRRLRTRSRPN | 237 |
| PAX2 | 113 | gst-TNAKHSSHN | 238 |
| PAX2 | 114 | gst-SSHNRRRLRTR | 239 |
| PAX2 | 115 | gst-RRRLRTRSRPN | 240 |
| SNi10 | 96 | gst-RVGQCTSDVRRPPWARS CAHQCGAGTRNSHGCTRPLRQASAH | 241 |
| SNi10 | 116 | gst-RVGQCTSDVRRPPWARS CA | 242 |
| SNi10 | 117 | gst-VRRPPWARS CAHQCGAGTRNS | 243 |
| SNi10 | 118 | gst-GTRNSHGCTRPLRQASAH | 244 |

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|------|-----|---|-----|
| DCX8 | 95 | gst-RYKHDI GCDAGVDKKSSSVRGGCGAHSSPPRAGRGRGTMVSR | 245 |
| DCX8 | 107 | gst-RYKHDI GCDAGVDKKSSSVRGGCG | 246 |
| DCX8 | 108 | gst-GCDAGVDKKSSSVRGGCGAHSSPPRA | 247 |
| DCX8 | 109 | gst-GAHSSPPRAGRGRGTMVSR | 248 |

6.10.6. Peptide Stability

The relative stability for ZElan031 (SEQ ID NO:297), ZElan053 (SEQ ID NO:319) and ZElan054 (SEQ ID NO:320) was determined in simulated intestinal fluid (SIF). SIF was made by dissolving 100mg of pancreatin (Sigma cat#P-1625, lot# 122H0812) in 8.4ml of phosphate stock solution, adjusting the pH to 7.5 with 0.2N NaOH and adjusting the volume to 10ml with water.

Peptide (3.25mg) was dissolved in 3.25 ml of 10,000 fold diluted SIF solution at 37°C. Aliquots (0.7ml) of the digestion solution were then withdrawn at <1min, 1h, 3h, and 21h or 24h. The samples were quickly passed through a syringe filter (Millipore Millex-GV 0.22µm, part# SLGV025LS, lot# H2BM95250) and 300µL of the filtered solution was immediately injected onto a Hewlett-Packard HPLC system equipped with a C-8 column (Applied Biosystems column and guard column: column- p/n 0711-0023 Spheri-5 ODS 5µm, 220x4.6mm). The products were eluted at 1.5ml/min using an acetonitrile-water gradient. The major fluorescent peaks were collected, lyophilized and identified by MS analysis.

The HPLC gradient used was:

| Time (min) | Solvent Mixture |
|---------------|---|
| 0 | 95% H ₂ O-5% acetonitrile (0.1%TFA) |
| 5 | 95% H ₂ O-5%acetonitrile (0.1%TFA) |
| 25 35 | 85% H ₂ O-15% acetonitrile (0.1%TFA) linear solvent change |
| 40 | 0% H ₂ O-100% acetonitrile (0.1%TFA) " |
| 45 | 95% H ₂ O-5% acetonitrile (0.1%TFA) " |
| 52 | 95% H ₂ O-5%acetonitrile (0.1%TFA) " |

As shown in Table 22, the relative stability (to SIF) for the three peptides was found to be

((

ZElan053>ZElan054>ZElan031 (SEQ ID NOs:319,320,297,
respectively). Enzymatic cleavage of the peptide was found to
occur at arginine and/or lysine as expected. The replacement
of l-amino acids with their D-amino acid analogs
5 significantly reduced the rate of proteolysis at these
residues.

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TABLE 22

| | <u>Peptide</u> | <u>Percent Remaining at:</u> | | | | <u>Rel. Stab.</u> | <u>SEQ ID NO</u> |
|---|----------------|------------------------------|------------|------------|-------------|-------------------|------------------|
| | | <u>1 m</u> | <u>1 h</u> | <u>3 h</u> | <u>24 h</u> | | |
| 5 | ZElan031 | 100 | 38.7 | 0 | 0 | 3 | 297 |
| | ZElan054 | 97.4 | 58.2 | 11.6 | 2.7 | 2 | 320 |
| | ZElan053 | 100 | 98.3 | 98.1 | 94.0 | 1 | 321 |

7. CHARACTERIZATION OF PEPTIDE-COATED PARTICLES

10 Binding of Peptide-Coated PLGA Nanoparticles to Fixed Caco-2 Cells

Binding of nanoparticles coated with targeting peptides to fixed Caco-2 cells was investigated using an ELISA assay based on reaction of antibody with the dansyl moiety present on the peptides. Isoelectric points of selected synthetic peptides are shown in Table 23 (corresponding SEQ ID NOS. are shown in Table 7). Corresponding dansylated synthetic GIT binding peptides are given in Table 24.

TABLE 23

| 20 | <u>Peptide</u> | <u>Sequence</u> | <u>pI</u> | <u>SEQ</u> |
|----|----------------|--|-----------|---------------|
| | | | | <u>ID NO.</u> |
| | P31 | SARDSGPAEDGSRVRLNGVENANTRKSSRSNPRGRRHP | 12.26 | 43 |
| | 5PAX5 | RGSTGTAGGERSGVLNLHTRDNASGSGFKPWYPSNRGHK | 11.49 | 46 |
| 25 | SNi10 | RVGQCTDSVRRPWARSCAHQCGAGTRNSHGCITRPLRQASAH | 10.45 | 4 |
| | SNi34 | SPCGGSWGRFMQGGFLGGRTDGC GAHRNRTSASLEPPSSDY | 8.25 | 6 |
| | DCX11 | SQGSKQCMQYRTGRLTVGSEYCGMNPARGHATPAYPARLLPRYR | 10.44 | 24 |
| | DCX8 | RYKHDIGCDAGVDKKSSSVRGCGAHSSPPRAGRGPRGTMVSRL | 11.03 | 23 |
| | HAX42 | SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVFNRRRPSAIP | 9.62 | 52 |
| | PAX2 | STPPSREAYSRPYSVSDSDTNAKHSSHNRRRLRTRSRPN | 11.26 | 55 |

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TABLE 24

| <u>Peptide</u> | <u>Sequence</u> | SEQ ID NO. |
|----------------|---|------------------|
| 5 P31 | H ₂ N-K(dns) SARDSGPAEDGSRAVRLNGVENANTRKSSRSNPRGRRHPGG-CONH ₂ | 288 |
| 5PAX5 | H ₂ N-K(dns) RGSTGTAGGERSGVLNLHTRDNASGSGFKPWYPSNRGHK-CONH ₂ | 282 |
| SNi10 | H ₂ N-K(dns) RVGQCTDSDVRRPWARSCAHQCGAGTRNSHGCITRPLRQASAH-CONH ₂ | 278 |
| SNi34 | H ₂ N-K(dns) SPCGGSWGRFMQGGFLFGGRTDGC GAHRNRTSASLEPPSSDY-CONH ₂ | 286 |
| DCX11 | H ₂ N-K(dns) SQGSKQCMQYRTGRLTVGSEYGC GMNPARHATPAYPARLLPRYR-CONH ₂ | 277 |
| DCX8 | H ₂ N-K(dns) RYKHDIGCDAGVDKSSSVRGCGAHSSPPRAGRGPRTMVSR-CONH ₂ | 287 |
| 10 HAX42 | H ₂ N-K(dns) SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVFNRRRPSAIPT-CONH ₂ | 285 |
| PAX2 | H ₂ N-K(dns) STPPSREAYSRPYSVSDSDTNAKHSSHNRLRTRSRPNG-CONH ₂ | 281 |
| DAB10 | H ₂ N-K(dns) SKSGEGGDSSRGETGWARVRSHAMTAGRFRWYNQLPSDR-CONH ₂ | 289 |

Method:

Confluent Caco-2 monolayers grown in 96-well plates (p38) were fixed and treated with 0.1% phenylhydrazine before blocking with 0.1% BSA in PBS. Control and dansyl peptide-coated nanoparticles were resuspended in sterile water at 10mg/ml and stirred with a magnet for 1h at room temperature. Samples consisted of: (1) blank nanoparticle control, (2) scrambled PAX2-coated nanoparticles, (3) PAX2-coated nanoparticles, (4) HAX42-coated nanoparticles, (5) PAX2/HAX42-coated nanoparticles, and (6) 8 peptide-coated nanoparticles.

Nanoparticles were added to the cells at 10mg/ml in 100μl 1%BSA-PBS (no Tween80 is used in this assay) and 2-fold serially-diluted. The 96-well plates were incubated for 1h at room temperature. The plates were washed 5 times with 1%BSA-PBS and 100μl of anti-dansyl antibody (Cytogen DB3-226.3; 0.5 μg/ml; batch May 1997) was added per well and the plates incubated 1h at room temperature. The wells were washed 5 times with 1%BSA-PBS; 100μl of goat anti-mouse λ:HRP antibody (Southern Biotechnology CN. 1060-05; 1:10,000) was added per well, and the plates incubated 1h at room temperature. After washing 5 times with 1%BSA-PBS, 100μl of

TMB peroxidase substrate (KPL CN. 50-76-00) was added to the wells and the optical density at 650nm was measured after 15 minutes.

As shown in Figures 13A-B, a decreasing anti-dansyl ELISA response was observed for nanoparticles coated with PAX2, HAX2, PAX2+HAX2, and a mixture of 8 targeting peptides, when decreasing amounts of the nanoparticles were applied to fixed Caco-2 cells. No concentration effect was observed for blank nanoparticles or nanoparticles coated with a scrambled version of PAX2 peptide. Nanoparticles coated with PAX2, HAX2, PAX2+HAX2, and the 8 peptide mix, showed increased response relative to blank nanoparticles or nanoparticles coated with a scrambled version of PAX2 peptide. The OD values were low relative to those normally observed for GST-peptide fusion binding to fixed Caco-2 cells.

Table 25 below shows the insulin potency and level of peptides coated onto the particles (measured by fluorescence) for formulation 1 particles (formulation by the coacervation method given below).

Table 25

| 20 | Peptide | Blend | |
|----|----------------|-----------------|------------------|
| | | Insulin mg/g | Peptide μl/mg |
| | PAX2 | 60.7 | 3.51 |
| | HAX42 | 55.9 | 2.93 |
| | PAX2 SCRAMBLED | 57.7 | 1.26 |
| | P31 | 67.0 | 1.22 |
| 25 | 5PAX5 | 52.7 | 2.83 |
| | SNi10 | 59.5 | 1.75 |
| | SNi34 | 61.5 | 4.03 |
| | DCX8 | 59.1 | 1.87 |
| | DAB10 | 55.9 | 1.99 |

ELISA of dansylated peptides and insulin coated PLGA particles

The standard ELISA procedure was modified as follows. Peptides and particles were diluted to an appropriate concentration in PBS containing 1%BSA (particles were sonicated to achieve a homogeneous solution), titered and incubated one hour at room temperature. Following five washes with PBS containing 1%BSA, an in-house IgG1λ anti-dansyl monoclonal antibody was added (diluted to 1μg/ml in 1%BSA-PBS) and the plates were incubated for one hour. After five more washes goat anti-mouse λ-HRP was added (Southern Biotechnology Associates Inc., Birmingham, AL, diluted 1:10,000 in 1%BSA-PBS) and the plates were incubated one hour. After five washes, plates were developed with TMB peroxidase substrate (Kirkegard and Perry, Gaithersburg, MD). All data is presented with background binding subtracted. Tween 20 was not added to the diluent or the washes when insulin coated PLGA particles were included in the assay.

Figures 14A-14B show the binding of the dansylated peptide SNI10 to hSI and BSA.

8. BINDING OF SYNTHETIC PEPTIDES AND PEPTIDE-COATED PARTICLES TO S100 AND P100 FRACTIONS DERIVED FROM CACO-2 CELLS

8.1. Detection of Binding to Membrane (P100) and Cytosolic (S100) fractions

Caco-2 cell membrane (P100) and cytosolic (S100) fractions were prepared using a modification of the method described in Kinsella, B. T., O'Mahony, D. J. and G. A. FitzGerald, 1994, J. Biol. Chem. 269(47): 29914-29919. Confluent Caco-2 cell monolayers (grown in 75 cm² flasks for up to 1 week at 37°C and 5% CO₂) were washed twice in Dulbecco's PBS (DPBS) and the cells were harvested by centrifugation at 1000 rpm after treatment with 10 mM EDTA-DPBS. The cells were washed 3 times in DPBS and the final cell pellet was resuspended in 3 volumes of ice cold

HED buffer (20 mM HEPES (pH 7.67), 1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride (PMSF)). The cells were allowed to swell for 5 min on ice prior to homogenization for 30 sec. The homogenates were centrifuged at 40,000 rpm for 45 min at 4°C. The supernatant (S100) was removed and the pellet (P100) was resuspended in HEDG buffer (20 mM HEPES (pH 7.67), 1 mM EGTA, 0.5 mM dithiothreitol, 100 mM NaCl, 10% glycerol, 1 mM PMSF). Protein concentrations were determined using the Bradford assay (Bradford, M. M., 1976, Anal. Biochem. 72: 248-254).

Binding of peptide and/or peptide-coated PLGA particles to membrane (P100) and cytosolic (S100) fractions was assessed by detection of the dansyl moiety incorporated in the peptide. Costar ninety six well ELISA plates were coated with S100 and P100 fractions (100 µg/ml in 0.05 M NaHCO₃) overnight at 4°C. The plates were blocked with 0.5% bovine serum albumin in DPBS for 1 h at room temperature and washed 3 times in 1% BSA-DPBS. Peptide-coated particles or peptides were dispersed in the same buffer and added to the plates at concentrations in the range 0.0325 - 0.5 mg/well. After 1 h at room temperature the plates were washed 5 times in 1% BSA-DPBS and 100 µl of anti-dansyl antibody (Cytogen DB3-226.3; 0.5 µg/ml) was added per well. The plates were incubated for 1 h at room temperature. The wells were washed 3 times in 1% BSA-DPBS and 100 µl of goat anti-mouse IgGλ:HRP antibody (Southern Biotechnology 1060-05; 1:10,000) was added per well. The plates were incubated for 1 h at room temperature. After washing 3 times in 1% BSA-DPBS 100 µl of TMB substrate (3,3',5',5'-tetramethylbenzidine; Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories 50-76-00)) was added and the optical density was measured at 650 nm at various time intervals.

8.2. Binding of Peptide-Coated PLGA particles

A novel assay system is provided by the instant invention for detection of binding of peptide-coated PLGA

particles to membrane (P100) and cytosolic (S100) fractions derived from live Caco-2 cells. The absorbance readings obtained using this assay system were substantially higher than those obtained using similar peptide-coated PLGA particle concentrations on fixed Caco-2 cells. This greater
5 sensitivity together with the derivation of the S100 and P100 fractions from live Caco-2 cells suggests that this assay may be the assay system of choice for detection of peptide-coated PLGA particle binding. The assay was concentration dependent and peptide/particle correlation permitted differentiation between specific and non-specific binding interactions.

10 Binding of peptide-coated PLGA particles was assessed using S100 and P100 fractions derived from live Caco-2 cells as described above. The fractions were coated onto 96-well plates at 10µg/well in 0.05 M NaHCO₃ and peptide-coated PLGA particles were assayed by ELISA at concentrations in the range 0.0325 - 0.5 mg/well.

15 Figures 15A and 15B illustrate the data obtained on S100 and P100 fractions respectively for particles coated with no peptide, scrambled PAX2 (control), P31 D-Arg 16-mer (ZElan053 , SEQ ID NO: 319), HAX42, PAX2 and HAX42/PAX2. Using particle concentrations of 0.0325 - 0.5 mg/well all test peptide-coated PLGA particles exhibited greater binding
20 to both the S100 and P100 fractions than the scrambled PAX2 coated control particles. All particles except P31 D-Arg 16-mer (ZElan053 , SEQ ID NO: 319) exhibited greater binding to the P100 fraction than the S100 fraction. Greater binding of the P31 D-Arg 16-mer (ZElan053 , SEQ ID NO: 319) coated particles to the S100 fraction may be indicative of
25 non-specific binding due to the D-Arg modification of the P31 peptide (SEQ ID NO: 270).

Binding of PLGA particles coated with varying concentrations of PAX2 peptide ranging from 0.05 - 5.0 mg/g was assessed using a) fixed Caco-2 cells (P35) and b) S100 and P100 fractions (Caco-2 P33). The particles were assayed
30 at concentrations in the range 0.03125 - 0.0625 mg/well.

Using a particle concentration of 0.0625 mg/well, all PAX2 coated particles except those coated at 0.05 mg/g exhibited greater binding to fixed Caco-2 cells than the scrambled PAX2 coated control particles. There appeared to be a concentration effect with increasing PAX2 peptide

5 concentration resulting in improved Caco-2 cell binding (in the range 0.05 - 1.0 mg/g). However all absorbance readings were low and binding of the PAX2 (5 mg/g) was not consistent with this pattern.

Using particle concentrations of 0.03125 - 0.0625 mg/well all test peptide coated particles except PAX2 (0.05
10 mg/g) exhibited comparable or greater binding to both the S100 and P100 fractions than the scrambled PAX2 coated control particles. All particles exhibited greater binding to the P100 fraction than the S100 fraction. Binding to both the S100 and P100 fractions was directly proportional to the concentration of the PAX2 peptide on the particle. The
15 absorbance readings obtained using this assay system were substantially higher than those obtained on the fixed Caco-2 cells.

The effect of blocking solution on binding of peptide-coated PLGA particles to P100 fractions (Caco-2 P35) was assessed using 1% bovine serum albumin (BSA) and 1% milk
20 powder blocking solutions to assess background binding. The following particles were assayed at concentrations in the range 0.03125 - 0.0625 mg/well: no peptide; scrambled PAX2; and a range of PAX2 coated particles having peptide concentrations from 5-0.05 mg/g. As previously observed using 1% BSA, all test peptide coated particles except PAX2 coated
25 at 0.05 mg/g exhibited comparable or greater binding to the P100 fractions than the scrambled PAX2 coated control particles. Binding to P100 fractions was directly proportional to the concentration of the PAX2 peptide on the particle (although in this instance PAX2 (5 mg/g) exhibited slightly lower binding than PAX2 (1 mg/g)). A similar trend
30 was observed using 1% milk powder and a particle concentration of 0.0625 mg/well. However all absorbance

readings were low when 1% milk powder was used and the binding pattern was not detectable using particles at a concentration of 0.0625 mg/well.

Non-specific binding of peptide-coated PLGA particles to plastic was also assessed using 1% BSA and 1% milk powder blocking solutions. The binding pattern observed above could be detected when BSA was used; however, absorbance readings were substantially lower and binding of particles PAX2 (0.1 and 0.05 mg/g respectively) was not detectable. When 1% milk powder was used, all absorbance readings were low and no binding pattern was detectable. BSA was chosen for blocking in subsequent assays.

8.3. Comparison of Peptide-Coated Particle and Synthetic Peptide Binding to P100 fractions

Binding of dansylated peptides to P100 fractions was assessed to determine if peptide binding was predictive of peptide-coated particle binding. Figure 16 illustrates the data obtained for the dansylated peptides A) HAX42, P31 D-form and scrambled PAX2 and B) PAX2, HAX42 and scrambled PAX2.

Two consecutive assays produced substantial variations in absorbance readings. Initially, the HAX42 peptide exhibited strong binding when compared to the scrambled PAX2 control. The P31 D-form peptide (ZElan053, SEQ ID NO: 319) exhibited binding at the highest dilution only. In the repeat assay, HAX42 also exhibited significant binding compared to the scrambled PAX2 control. However, the scrambled PAX2 control and HAX42 produced relatively high absorbance values compared to those obtained in the previous assay. The PAX2 peptide was indistinguishable from the scrambled PAX2 control. Peptide/particle binding correlation is summarized as follows in Table 26:

TABLE 26

Peptide/particle assay correlation

| | Peptide | Assay correlation |
|---|---------------------------------------|-------------------|
| | HAX42 | + |
| | PAX2 | +/- |
| | P31 D-form | - |
| | Scrambled | +/- |
| | PAX2 | |
| 5 | + positive; +/- equivocal; - negative | |

Peptide/particle binding correlated well for the HAX42 peptide. In contrast, no correlation could be detected for the P31 D-form (ZElan053 , SEQ ID NO: 319) peptide. Since the P31 D-form peptide-coated particles exhibited greater
10 binding to the S100 fraction than the P100 fraction (unlike the other test peptides) it appears that the particle binding interaction was non-specific or that some other molecule was competing for binding to the P100 fraction but not to the S100 fraction. Thus the peptide/particle assay correlation may be useful for distinguishing between specific and
15 non-specific binding interactions. The scrambled PAX2 control produced variable results so that it was difficult to assess the PAX2 binding correlation.

8.4. Determination of HAX42 and PAX2 Binding Motif Sequences

20 Peptides and GST fusion proteins of HAX42, PAX2 and various derivatives were assayed using peptide ELISA to P100 membrane fractions derived from Caco-2 cells. The GST-PAX2 protein and PAX2 peptide data indicate that a core binding motif lies in the amino acid sequence TNAKHSSHNRRLRTR (SEQ ID NO: 402) otherwise named GST-106 and ZElan033 (SEQ ID NO:
25 299). Similarly, the HAX42 peptide data suggest that a core binding motif for HAX42 lies in the amino acid sequence PGDYNCCGNCNSTG (SEQ ID NO: 403), otherwise named ZElan091 (SEQ ID NO: 358).

The peptides and proteins were analyzed by a dansylated peptide ELISA method in which 96 well plates were
30 coated overnight at 4°C with 100µl/well coating protein (normally 100µg/ml P100 membrane fraction) in 0.05M carbonate

buffer pH9.6. Nonspecific binding was blocked using 200 μ l/well, 2% Marvel/PBS for 2 hours at 37°C prior to incubation with dansylated peptides. The plates were washed three times with PBS/0.05% Tween 20 and after each subsequent incubation step. The peptides were diluted in blocking solution at a starting concentration of 100 μ g/ml and diluted 1:2 downwards, 100 μ l/well, followed by incubation at room temperature for 1 hour, exactly. A buffer blank control was included to ensure that background binding to plastic was not due to the antibodies used in the assay system. To detect the dansylated peptides, a mouse anti-dansyl antibody (DB3, Cytogen Corp.) at 1:1340 dilution in blocking buffer and 100 μ l/well was added followed by incubation at room temperature for 1 hour. The plates were then incubated with an anti-mouse λ -HRP conjugated antibody (Southern Biotech 1060-05) at a 1:10,000 dilution in blocking solution, 100 μ l/well for 1 hour at room temperature. Plates were developed using 75 μ l/well Bionostics TMB substrate and incubated for approximately 10 minutes. The developing reaction was stopped using Bionostics Red Stop solution (25 μ l/well), and the optical density of the plates was read at 650nm.

20 GST-PAX2 Peptides - Relative Binding to P100 Fractions

After subtraction of the GST-peptide binding to plastic from P100 binding values, the binding of GST-PAX2 peptides were represented as a ratio of GST-HAX42 binding to P100, which was given the arbitrary value of 1.00. The following ratios were determined from binding to P100 of GST-peptides at a peptide concentration of 20 μ g/ml. Bold denotes positive binding to the P100 membrane fraction.

Table 27

| | GST-peptide | Value |
|----|------------------|-------------|
| | GST-HAX42 | 1.00 |
| | GST-PAX2 | 1.79 |
| 30 | GST-104 | 0.01 |
| | GST-105 | -0.08 |

| | |
|----------------|-------------|
| GST-106 | 2.71 |
| GST-113 | 0.26 |
| GST-114 | 0.17 |
| GST-115 | 0.36 |
| GST | 0.48 |

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Table 28

| GST-peptide Amino Acid Sequence | | SEQ. ID. No. |
|---------------------------------|---|-----------------|
| GST-PAX2 | STPPSREAYSRPYSVDSDSDTNAKHSSHNRRRLRTRSRPN | 55 |
| GST-104 | STPPSREAYSRPYSVDSDSD | 170 |
| GST-105 | STPPSREAYSRPYSVDSDSDTNAKHSSHN | 171 |
| 5 GST-106 | TNAKHSSHNRRRLRTRSRPN | 172 |
| GST-113 | TNAKHSSHN | 173 |
| GST-114 | SSHNRRRLRTRSRPN | 174 |
| GST-115 | RRLRTRSRPN | 175 |

PAX2 Peptides - Relative Binding to P100 Fractions

10 ZElan021 (SEQ ID NO: 285), full length HAX42, was given the arbitrary value of 1.00 for binding to P100 at a given peptide concentration determined from the signal-to-noise ratio data. PAX2 and its derivatives are given as a ratio of HAX42 value to reflect their binding abilities to P100 membrane fractions derived from a Caco-2 cell line as shown

15 in Table 329. Table 30 provides a line-up of the PAX2 peptides showing the positive binding peptides in boldface. The GST-PAX2 peptide and PAX2 peptide data agree, demonstrating that a binding motif is in the amino acid sequence **TNAKHSSHNRRRLRTR** (SEQ ID NO: 402) (GST-106 and ZElan033, SEQ ID NO: 299) .

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TABLE 29

| | SEQ ID NO. | PAX2 peptide | Binding value at 20µg/ml | Binding value at 20µg/ml | Binding value at 50µg/ml | Binding value at 50µg/ml | Binding value at 50µg/ml (Jackso n Ab) | Binding value at 50µg/ml (Southe rn Ab) |
|-----------|------------------|-----------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|---|--|
| 5 | | | | | | | | |
| | 281 | ZElan018 | -0.33 | 1.07 | 0.95 | 1.01 | | |
| | 298 | ZElan032 | 1.43 | 2.87 | 0.95 | 1.06 | | |
| | 299 | ZElan033 | 0.35 | 1.57 | 0.80 | 0.66 | | |
| | 301 | ZElan035 | 0.12 | 0.43 | 0.81 | 0.77 | | |
| | 321 | ZElan055 | 0.99 | 0.73 | 1.10 | 0.59 | | |
| | 322 | ZElan056 | 0.00 | 0.16 | 0.21 | 0.21 | | |
| | 323 | ZElan057 | 0.08 | | 0.56 | 0.25 | | |
| 10 | 324 | ZElan058 | 0.05 | | 0.47 | 0.16 | | |
| | 339 | ZElan073 | 0.07 | | -0.11 | 0.49 | 0.66 | 0.49 |
| | 340 | ZElan074 | 0.06 | | 0.82 | 0.52 | 0.71 | 0.48 |
| | 341 | ZElan075 | 0.13 | | 0.52 | 0.38 | 0.47 | 0.32 |
| | 342 | ZElan076 | 0.08 | | 1.00 | 0.41 | 0.60 | 0.42 |
| | 343 | ZElan077 | 0.20 | | 0.76 | 0.54 | 0.73 | 0.52 |
| | 344 | ZElan078 | 0.11 | | 0.87 | 0.69 | 0.68 | 0.47 |
| | 345 | ZElan079 | 0.31 | | 0.97 | 0.68 | 0.83 | 0.53 |
| | 346 | ZElan080 | 0.23 | | 0.84 | 0.45 | 0.67 | 0.38 |
| 15 | 347 | ZElan081 | 0.01 | | 0.89 | 0.47 | | |
| | 348 | ZElan082 | 0.00 | | 0.92 | 0.40 | | |
| | 350 | ZElan083 | 0.43 | 0.63 | 1.03 | 0.88 | | |
| | 351 | ZElan084 | 1.06 | 0.93 | 1.16 | 0.77 | | |

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Table 30

| | PAX2 Peptide | Amino acid sequence | SEQ ID NO: |
|----|--------------------------|--|---------------|
| | ZElan018 | H ₂ N-K (dns) STPPSREAYSRPYSVSDSDDTNAKHSSHNRRLRTRSRPNG -CONH ₂ | 281 |
| | ZElan032 | H ₂ N-K (dns) TNAKHSSHNRRLRTRSRPN-CONH ₂ | 298 |
| | ZElan033 | H ₂ N-K (dns) TNAKHSSHNRRLRTR-CONH ₂ | 299 |
| 5 | ZElan034 | H ₂ N-K (dns) SSHNRRLRTRSRPN-CONH ₂ | 300 |
| | ZElan035 | H ₂ N-K (dns) SSHNRRLRTR-CONH ₂ | 301 |
| | ZElan055 | H ₂ N-K (dns) TNAKHSSHNRRLRTR-CONH ₂ | 321 |
| | ZElan056 | H ₂ N-K (dns) RRLRTRSRPN-CONH ₂ | 322 |
| | ZElan057 | H ₂ N-K (dns) RRLRTRSR-CONH ₂ | 323 |
| | ZElan058 | H ₂ N-K (dns) RRLRTR-CONH ₂ | 324 |
| | ZElan059 | H ₂ N-K (dns) rrLrTrSrPN-CONH ₂ | 325 |
| | ZElan073 | H ₂ N-K (dns) ASHNRRLRTR-CONH ₂ | 339 |
| | ZElan074 | H ₂ N-K (dns) SAHNRRLRTR-CONH ₂ | 340 |
| 10 | ZElan075 | H ₂ N-K (dns) SSANRRLRTR-CONH ₂ | 341 |
| | ZElan076 | H ₂ N-K (dns) SSHARRLRTR-CONH ₂ | 342 |
| | ZElan077 | H ₂ N-K (dns) SSHNARLRTR-CONH ₂ | 343 |
| | ZElan078 | H ₂ N-K (dns) SSHNRALRTR-CONH ₂ | 344 |
| | ZElan079 | H ₂ N-K (dns) SSHNRRARTR-CONH ₂ | 345 |
| | ZElan080 | H ₂ N-K (dns) SSHNRRLATR-CONH ₂ | 346 |
| | ZElan081 | H ₂ N-K (dns) SSHNRRLRAR-CONH ₂ | 347 |
| | ZElan082 | H ₂ N-K (dns) SSHNRRLRTA-CONH ₂ | 348 |
| | SCRAMBLED PAX2 PEPTIDES: | | |
| 15 | ZElan083 | H ₂ N-K (dns) GRNHVSSNTHKSYRSPRSASYPRLSNDRTDRTEPAPSS-CONH ₂ | 350 |
| | ZElan084 | H ₂ N-K (dns) RNTRNKTSRLSANPHRSHR-CONH ₂ | 351 |

HAX42 Peptides - Relative Binding to P100 Fractions

ZElan021 (SEQ ID NO: 285), full length HAX42, was given the arbitrary value of 1.00 for binding to P100 at a given peptide concentration determined from the signal-to-noise ratio data. HAX42 and its derivatives are given as a ratio of HAX42 value to reflect their binding abilities to P100 membrane fractions derived from a Caco-2 cell line as shown in Table 31. Table 32 provides a line-up of the HAX42 peptides showing the positive binding peptides in boldface.

A core binding motif appears to lie in the amino acid sequence PGDYNCCGNCNSTG (ZElan091, SEQ ID NO:358).

TABLE 31

| SEQ ID NO. | HAX42 peptide | Binding value at 20µg/ml | Binding value at 50µg/ml at 50µg/ml | Binding value at 50µg/ml | Binding value at 25µg/ml | Binding value at 25µg/ml | Binding value at 25µg/ml |
|------------------|------------------|--------------------------------|--|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| | 285 ZElan021 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| | 326 ZElan060 | 0.44 | 0.56 | 0.43 | | | |
| 5 | 327 ZElan061 | 0.20 | 0.60 | 0.38 | | | |
| | 328 ZElan062 | 0.11 | 0.42 | 0.34 | | | |
| | 331 ZElan065 | 0.00 | 0.54 | 0.30 | | | |
| | 333 ZElan067 | 0.08 | 0.52 | 0.40 | | | |
| | 336 ZElan070 | 0.59 | 0.97 | 0.39 | | | |
| | 337 ZElan071 | 1.22 | 0.89 | 0.75 | | | |
| | 338 ZElan072 | 0.83 | 0.61 | 0.88 | | | |
| | 354 ZElan087 | | | | 0.46 | 0.44 | |
| | 355 ZElan088 | | | | 2.21 | 1.41 | 1.63 |
| 10 | 356 ZElan089 | | | | 0.55 | 0.44 | 0.49 |
| | 357 ZElan090 | | | | 2.06 | 1.54 | 2.16 |
| | 358 ZElan091 | | | | 2.02 | 1.37 | 1.20 |
| | 359 ZElan092 | | | | 1.41 | 1.90 | 0.91 |
| | 360 ZElan093 | | | | 1.88 | 1.37 | 1.33 |

Table 32

| 15 | HAX42 | Amino acid sequence | SEQ. ID. NO. |
|----|----------|--|-----------------|
| | Peptide | | |
| | ZElan021 | H ₂ N-K (dns) SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVFNRRRPSAIP-CONH ₂ | 285 |
| | ZElan060 | H ₂ N-K (dns) SDHALGTNLRSDNAKEPGDYNCCGNG-CONH ₂ | 326 |
| | ZElan061 | H ₂ N-K (dns) GNGNSTGRKVFNRRRPSAIP-CONH ₂ | 327 |
| | ZElan062 | H ₂ N-K (dns) SDHALGTNLRSDNAKEPG-CONH ₂ | 328 |
| | ZElan065 | H ₂ N-K (dns) RKVFNRRRPS-CONH ₂ | 331 |
| | ZElan067 | H ₂ N-K (dns) NRRRPS-CONH ₂ | 333 |
| 20 | ZElan070 | H ₂ N-K (dns) SDHALGTNLRSDNAKEPGDYNCCGNGNST-CONH ₂ | 336 |
| | ZElan071 | H ₂ N-K (dns) NLRSDNAKEPGDYNCCGNGNSTGRKVFNR-CONH ₂ | 337 |
| | ZElan072 | H ₂ N-K (dns) PGDYNCCGNGNSTGRKVFNRRRPSAIP-CONH ₂ | 338 |
| | ZElan087 | H ₂ N-K (dns) SDHALGTNLRSDNAKEPGDY-CONH ₂ | 354 |
| | ZElan088 | H ₂ N-K (dns) SDNAKEPGDYNCCGNGNSTG-CONH ₂ | 355 |
| | ZElan089 | H ₂ N-K (dns) SDHALGTNLRSDNAK-CONH ₂ -CONH ₂ | 356 |
| | ZElan090 | H ₂ N-K (dns) EPGDYNCCGNGNSTG | 357 |
| | ZElan091 | H ₂ N-K (dns) PGDYNCCGNGNSTG-CONH ₂ | 358 |
| | ZElan092 | H ₂ N-K (dns) PGDYNCCGNG-CONH ₂ | 359 |
| 25 | ZElan093 | H ₂ N-K (dns) NCCGNGNSTG-CONH ₂ | 360 |

9. FORMULATIONS

General Method for Preparation of Coacervated Particles.

Solid particles containing a Therapeutic as defined
 30 herein are prepared using a coacervation method. The are
 particles are formed from a polymer and have a particle size of

between about 10nm and 500 μ m, most preferably 50 to 800 nm. In addition the particles contain targeting ligands which are incorporated into the particles using a number of methods.

The organic phase (B) polymer of the general method given above may be soluble, permeable, impermeable, 5 biodegradable or gastroretentive. The polymer may consist of a mixture of polymer or copolymers and may be a natural or synthetic polymer. Representative biodegradable polymers include without limitation polyglycolides; polylactides; poly(lactide-co-glycolides), including DL, L and D forms; copolyoxalates; polycaprolactone; polyesteramides; 10 polyorthoesters; polyanhydrides; polyalkylcyanoacrylates; polyhydroxybutyrates; polyurethanes; albumin; casein; citosan derivatives; gelatin; acacia; celluloses; polysaccharides; alginic acid; polypeptides; and the like, copolymers thereof, mixtures thereof and stereoisomers thereof. Representative synthetic polymers include alkyl celluloses; hydroxalkyl 15 celluloses; cellulose ethers; cellulose esters; nitrocelluloses; polymers of acrylic and methacrylic acids and esters thereof; dextrans; polyamides; polycarbonates; polyalkylenes; polyalkylene glycols; polyalkylene oxides; polyalkylene terephthalates; polyvinyl alcohols; polyvinyl ethers; polyvinyl esters; polyvinyl halides; 20 polyvinylpyrrolidone; polysiloxanes and polyurethanes and co-polymers thereof.

Typically, particles are formed using the following general method:

An aqueous solution (A) of a polymer, surface active agent, surface stabilising or modifying agent or salt, or 25 surfactant preferably a polyvinyl alcohol (PVA) or derivative with a % hydrolysis 50 - 100% and a molecular weight range 500 - 500,000, most preferably 80-100% hydrolysis and 10,000-150,000 molecular weight, is introduced into a vessel. The mixture (A) is stirred under low shear conditions at 10-2000 rpm, preferably 100-600 rpm. The pH and/or ionic strength 30 of this solution may be modified using salts, buffers or other modifying agents. The viscosity of this solution may be

((

modified using polymers, salts, or other viscosity enhancing or modifying agents.

A polymer, preferably poly(lacide-co-glycolide), polylactide, polyglycolide or a combination thereof or in any enantiomeric form or a covalent conjugate of the these polymers
5 with a targeting ligand is dissolved in water miscible organic solvents to form organic phase (B). Most preferably, a combination of acetone and ethanol is used in a range of ratios from 0:100 acetone: ethanol to 100: 0 acetone: ethanol depending upon the polymer used.

Additional polymer(s), peptide(s) sugars, salts,
10 natural/biological polymers or other agents may also be added to the organic phase (B) to modify the physical and chemical properties of the resultant particle product.

A drug or bioactive substance may be introduced into either the aqueous phase (A) or the organic phase (B). A targeting ligand may also be introduced into either the aqueous
15 phase (A) or the organic phase (B) at this point.

The organic phase (B) is added into the stirred aqueous phase (A) at a continuous rate. The solvent is evaporated, preferably by a rise in temperature over ambient and/or the use of a vacuum pump. The particles are now present as a suspension (C). A targeting ligand may be introduced into
20 the stirred suspension at this point.

A secondary layer of polymer(s), peptide(s) sugars, salts, natural/biological polymers or other agents may be deposited on to the pre-formed particulate core by any suitable method at this stage.

The particles (D) are then separated from the
25 suspension (C) using standard colloidal separation techniques, preferably by centrifugation at high 'g' force, filtration, gel permeation chromatography, affinity chromatography or charge separation techniques. The supernatant is discarded and the particles (D) re-suspended in a washing solution (E) preferably water, salt solution, buffer or organic solvent(s). The
30 particles (D) are separated from the washing liquid in a

similar manner as previously described and re-washed, commonly twice. A targeting ligand may be dissolved in washing solution (E) at the final washing stage and may be used to wash the particles (D).

5 The particles may then be dried. Particles may then be further processed for example, tabletted, encapsulated or spray dried.

The release profile of the particles formed above may be varied from immediate to controlled or delayed release dependent upon the formulation used and/or desired.

Drug loading may be in the range 0-90% w/w.

10 Targeting ligand loading may be in the range 0-90% w/w.

Specific examples include the following examples:

EXAMPLE 1: Peptide added at the final washing stage

Product: Bovine Insulin loaded nanoparticles

15 **Aim:** To prepare a 2g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the peptide ZElan018 (SEQ ID NO: 281) added.

Formulation Details

| | | |
|---|------------------|-----------------------------|
| RG504H | (Lot no. 250583) | 2.0g |
| Acetone | | 45ml |
| 20 Ethanol: | | 5ml |
| PVA (aq. 5%w/v) | | 400ml |
| Bovine Insulin (Lot no. 86H0674) | | 100mg |
| Peptide: PAX2 (ZElan018 , SEQ ID NO: 281) | | 10mg/50ml dH ₂ O |

Experimental details:

25 The 5% w/v PVA solution was prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase was prepared by adding acetone, 45ml, and ethanol, 5ml, together. The polymer solution was prepared by adding RG504H, 2g, to the organic phase and stirring until dissolved. The IKA™ reactor vessel was set up, all seals
30 greased and the temperature was set at 25°C. The PVA solution,

400ml, was added into the reactor vessel and stirred at 400 rpm.

Bovine insulin, 100mg, was added into the stirring PVA solution. Using clean tubing and a green needle, the polymer solution was slowly dripped in the stirring PVA solution with
5 the peristaltic pump set at 40. The solvent was allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm.

The suspension was centrifuged in a Beckman Ultracentrifuge™ with swing-out rotor at 12,500 rpm, 4°C. The supernatant was decanted and discarded. The "cake" of
10 particles was broken up and dH₂O (200mls) was added to wash the particles. The centrifugation and washing steps were repeated twice.

The peptide solution, (ZElan018, SEQ ID NO: 281), 10mg in 50ml dH₂O) was prepared and added to the particles for a final washing stage. The suspended particles were centrifuged
15 as before. The supernatant liquid was decanted, the 'cake' broken up, and the particles were dried in the vacuum oven.

The particles were ground, placed in a securitainer and sent for analysis. The weight of particles recovered was 1.45g. A SEM showed discrete, reasonably spherical particles in the 300-500nm size range. The potency was 49.2mg/g (98.0%
20 of label claim). Peptide loading was 2.42 µg/mg (48.4% of label claim).

EXAMPLE 2: Peptide added at the beginning of manufacture

Product: Bovine Insulin loaded nanoparticles

25 **Aim:** To prepare a 2g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the peptide ZElan018 (SEQ ID NO: 281) added at the beginning of manufacture.

Formulation Details

| | | |
|------------|------------------|------|
| RG504H | (Lot no. 250583) | 2.0g |
| 30 Acetone | | 45ml |
| Ethanol: | | 5ml |

| | |
|----------------------------------|-------|
| PVA(aq. 5%w/v) | 400ml |
| Bovine Insulin (Lot no. 65H0640) | 100mg |
| Peptide: PAX2 (ZElan018ii) | 10mg |

Experimental details:

5 The 5% w/v PVA solution was prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase was prepared by adding acetone, 45ml, and ethanol, 5ml, together. The polymer solution was prepared by adding RG504H (polyactide-co-glycolide, Boehringer Ingelheim), 2g, to the organic phase prepared in step above and stirring
10 until dissolved. The IKA™ reactor vessel was set up, all seals greased and the temperature was set at 25°C. The PVA solution, 400ml, was added into the reactor vessel and stirred at 400 rpm.

Bovine insulin, 100mg, was added into the stirring PVA solution. PAX2 (ZElan018ii, 10mg) was added to the
15 stirring PVA solution. Using clean tubing and a green needle, the polymer solution was slowly dripped into the stirring PVA solution with the peristaltic pump set at 40. The solvent was allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm. The suspension was centrifuged in a Beckman Ultracentrifuge™ with swing-out rotor
20 at 12,500 rpm, 4°C. The supernatant was decanted and discarded.

The "cake" of particles was broken up and dH₂O (200ml) was added to wash the particles. The centrifugation and washing steps were repeated twice. The "cake" was broken up and the particles were dried in the vacuum oven.
25

The particles were ground, placed in a securitainer and sent for analysis. The weight of the particles recovered was 1.6g. The potency was 47.3mg/g (94.6% of label claim). Peptide loading was 1.689µg/mg (33.8% of label claim).

EXAMPLE 3 Peptide added 1 hour before centrifugation
30 **Product:** Bovine Insulin loaded nanoparticles

Aim: To prepare a 1g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the peptide ZElan018 (SEQ ID NO: 281) added 1 hour before centrifugation.

Formulation Details

| | | |
|---|------------------|--------|
| RG504H | (Lot no. 250583) | 1.0g |
| 5 Acetone | | 22.5ml |
| Ethanol: | | 2.5ml |
| PVA(aq. 5%w/v) | | 200ml |
| Bovine Insulin (Lot no. 65H0640) | | 50mg |
| Peptide: PAX2 (ZElan018 , SEQ ID NO: 281) | | 5mg |

10 **Experimental details:**

The 5% w/v PVA solution was prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase was prepared by adding acetone, 22.5ml, and ethanol, 2.5ml, together. The polymer solution was prepared by adding RG504H, 1g, to the organic phase prepared above and stirring until dissolved. The IKA™ reactor vessel was set up, all seals greased and the temperature was set at 25°C. The PVA solution, 200ml, was added into the reactor vessel and stirred at 400 rpm.

Bovine insulin, 50mg, was added into the stirring PVA solution. Using clean tubing and a green needle, the polymer solution was slowly dripped in the stirring PVA solution with the peristaltic pump set at 40. The solvent was allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm.

PAX2 ((ZElan018, SEQ ID NO: 281) 5mg) was added to the stirring particle suspension. After 1 hr, the suspension was centrifuged in a Beckman Ultracentrifuge™ with swing-out rotor at 12,500 rpm, 4°C. The supernatant was decanted and discarded. The "cake" of particles was broken up and dH₂O (200ml) was added to wash the particles. The centrifugation and washing steps were repeated twice.

The 'cake' was broken up and the particles were dried in the vacuum oven. The particles were ground, placed in a

securitainer and sent for analysis. Potency was 20.75mg/g (41.5% of label claim). Peptide loading was 1.256µg/mg (25.12 % of label claim).

EXAMPLE 4: Leuprolide acetate loaded nanoparticles

- 5 **Aim:** To prepare a 3g batch of leuprolide-acetate loaded nanoparticles at a theoretical loading of 20mg/g and with the peptide ZElan024 (SEQ ID NO: 288) added.

Formulation Details

| | | |
|--|------------------|-----------|
| RG504H | (Lot no. 271077) | 3.0g |
| Acetone | | 67.5ml |
| 10 Ethanol: | | 7.5ml |
| PVA(aq. 5%w/v) | | 600ml |
| Leuprolide acetate | (Lot no. V14094) | 60mg |
| Peptide: P31 (ZElan024 , SEQ ID NO: 288) | | 15mg/50ml |
| dH ₂ O | | |

15 **Experimental details:**

The PVA solution was prepared and the organic phase was prepared by adding acetone, 67.5ml, and ethanol, 7.5ml, together. The polymer solution was prepared by adding RG504H, 3g, to the organic phase prepared above and stirring until dissolved. The IKA™ reactor vessel was set up, all seals greased and the temperature was set at 25°C. The PVA solution, 600ml, was added into the reactor vessel and stirred at 400 rpm.

25 Leuprolide acetate, 60mg, was added into the stirring PVA solution. Using clean tubing and a green needle, the polymer solution, was slowly dripped in the stirring PVA solution with the peristaltic pump set at 40. The solvent was allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm. The suspension was centrifuged in a Beckman Ultracentrifuge™ with swing-out rotor at 15,000 rpm, 4°C. The supernatant was decanted and retained for analysis.

30

The "cake" of particles was broken up and dH₂O 200ml) was added to wash the particles. The centrifugation and washing steps were repeated twice.

The peptide solution (P31 (SEQ ID NO: 270), 15mg in 50ml dH₂O) was prepared and added to the particles for a
5 final washing stage. The suspended particles were centrifuged as before. The supernatant liquid was decanted, and the particles were dried in the vacuum oven.

The particles were ground, placed in a securitainer and sent for analysis. The weight of particles recovered was 1.87g. SEM showed discrete, reasonably spherical particles in
10 the 300-500nm size range. The potency was 4.7mg/g (23.4% of label claim). Peptide loading was 1.76µg/mg.

EXAMPLE 5: Peptide added by 'spiking' polymer phase with polymer-peptide conjugate

15 **Product:** Bovine Insulin loaded nanoparticles

Aim: To prepare a 3g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the polymer-peptide conjugate PLGA-ZElan019 added.

Formulation Details

| | | |
|---------------------------------|-------------------|--------|
| RG504H | (Lot no. 271077) | 2.85g |
| 20 RG504H-ZElan019 conjugate | (5PAX5-conjugate) | 0.15g |
| Acetone | | 67.5ml |
| Ethanol: | | 7.5ml |
| PVA(aq. 5%w/v) | | 600ml |
| Bovine Insulin(Lot no. 86H0674) | | 150mg |

25

Experimental details:

The 5% w/v PVA solution was prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase was prepared by adding acetone, 67.5ml, and ethanol, 7.5ml, together. The polymer solution was prepared by
30

adding RG504H and the polymer-peptide conjugate to the organic phase and stirring until dissolved.

The IKA™ reactor vessel was set up, all seals greased and the temperature was set at 25°C. The PVA solution, 400ml, was added into the reactor vessel and stirred at 400
5 rpm.

Bovine insulin, 100mg, was added into the stirring PVA solution. Using clean tubing and a green needle, the polymer solution, was slowly dripped in the stirring PVA solution with the peristaltic pump set at 40. The solvent was allowed to evaporate by opening the ports and allowing the
10 dispersion to stir overnight at 400 rpm.

The suspension was centrifuged in a Beckman Ultracentrifuge™ with swing-out rotor at 12,500 rpm, 4°C. The supernatant was decanted and discarded. The "cake" of particles was broken up and dH₂O (200ml) was added to wash the particles. The centrifugation washing step was repeated twice.
15

The 'cake' was broken up and the particles were dried in the vacuum oven. The particles were ground, placed in a securitainer and sent for analysis. The weight of particles recovered was 2.8g. The potency was 53.1mg/g 106.2% of label claim). Peptide loading was 4.02 µg/mg (80.4% of label claim).

20 10. ANIMAL STUDIES

Study 1

An open-loop study in which the test solution was injected directly into the ileum was done. Wistar rats (300-350g) were fasted for 4 hours and anaesthetized by
25 intramuscular administration 15 to 20 minutes prior to administration of the test solution with a solution of ketamine [0.525 ml of ketamine (100 mg/ml) and 0.875 ml of acepromazine maleate-BP ACP (2mg/ml)]. The rats were then injected with a test solution (injection volume: 1.5ml PBS) intra-duodenally at 2-3 cm below the pylorus. The test solution contained either
30 PLGA particles manufactured according to the coacervation procedure given above with or without targeting peptides or by

the "spiked" method given above. Insulin (fast-acting bovine; 28.1 iu/mg) was incorporated in the particles at 5% drug loading for a total of 100iu insulin (70 mg particles) or 300iu insulin (210 mg particles). Blood glucose values for the rats were measured using a Glucometer™ (Bayer; 0.1 to 33.3 m/mol/L); plasma insulin values were measured using a Phadeseph RIA Kit™ (Upjohn Pharmacia; 3 to 240 µU/ml-assayed in duplicate). Systemic and portal blood was sampled.

Study groups included animals receiving test solutions containing particles coated with the following peptides shown in Table 33.

10

Table 33

| | Study Group | Receptor | Peptide |
|----|--------------|----------|----------------------|
| | | | SNi10 |
| 15 | I | hSI | SNi34 |
| | II | hPEPT1 | P31 |
| | | | 5PAX5 |
| | III | HPT1 | PAX2 |
| | | | HAX42 |
| 20 | IV | D2H | DCX8 |
| | | | DCX11 |
| | V ("spiked") | hPEPT1 | P31-PLGA conjugate |
| | | | 5PAX5-PLGA conjugate |

Control groups included: 1) PBS control (1.5ml) Open-Loop; 2) Insulin solution (1iu/0.2ml) subcutaneous; 3) Insulin particles - no peptide (1iu/0.2ml) subcutaneous; 4) Insulin particles/all 8 peptides mix (1iu/0.2ml) subcutaneous; 5) Insulin loaded particles/peptide control (scrambled 5PAX5) (100iu/1.5ml) Open-Loop; 6) Insulin loaded particles/peptide control (scrambled 5PAX5) (300iu/1.5ml) Open-Loop; 7) Control particles (insulin-free)/all 8 peptide mix (equivalent 100iu/1.5ml) Open-Loop; and 8) Control particles (insulin-free)/all 8 peptide mix (equivalent 300iu/1.5ml) Open-Loop.

The following describes the pharmacokinetics for 300iu-loading:

| Target Receptor | F%** | Fold-increase** | Stat. Sig.** |
|------------------|-------|-----------------|--------------|
| HPT1 | 10.37 | 17.0 | <0.001 |
| Spiked hPEPT1 | 4.94 | 7.5 | 0.005 |
| 5 PAX2 scrambled | 3.50 | 3.6 | NS |
| Mix-8 | 2.00 | 2.0 | NS |
| hPEPT1 | 1.60 | 1.5 | NS |
| D2H | 1.57 | 1.4 | NS |
| hSI | 0.54 | 0.9 | NS |

* based on area under the curve (AUC) (1-4h), base-line adjusted, relative to subcutaneous insulin solution 1iu

** Fold increase in AUC compared to insulin particles: 300iu

10

Figures 17A and 17B show the systemic blood glucose and insulin levels following intestinal administration of control (PBS); insulin solution; insulin particles; all 8 peptides mix particles and study group peptide-particles (100iu). Figures 18A and 18B show the systemic blood glucose and insulin levels following intestinal administration of control (PBS); insulin solution; insulin particles and study group peptide-particles (300iu).

15

HPT1 targeted peptide coated particles provided the most potent enhancement of the delivery of insulin over subcutaneous injection of insulin followed by hPEPT1 spiked > PAX2 scrambled > mix-8 > hPEPT1 > D2H > uncoated particles > hSI > solution. In a repeat study, the uncoated particles containing insulin gave similar profiles but the HPT1-peptide targeted particles gave a reduced profile (3-fold). The insulin-free PLGA particles and the all-8 mix particles did not show an effect on the basal insulin or glucose levels. The HPT1 targeting particles, the PEPT1 spiked, targeting particles, and the PEPT1 targeting particles also reduced blood glucose levels indicative that the insulin delivered was bioactive. The other targeting particles were also shown to reduce blood glucose levels although not to the same extent as the HPT1 and PEPT1 spiked particles. No histological differences were observed in the small intestine for any of the formulations evaluated.

25

30

Study 2

A second open-loop study, similar to study 1 above, was undertaken with the following treatment groups as shown in Table 34.

5

Table 34

| | Group Number | Dose Insulin (iu) | Description |
|----|-----------------|-------------------------|--|
| | 1 | | PBS control |
| 10 | 2a | 1 | subcutaneous, bovine insulin |
| | 2b | 2 | subcutaneous, bovine insulin |
| | 2c | 3 | subcutaneous, bovine insulin |
| | 2d | 4 | subcutaneous, bovine insulin |
| | 2e | 10 | subcutaneous, bovine insulin |
| | 2f | 20 | subcutaneous, bovine insulin |
| | 2g | 4 | subcutaneous, human insulin |
| 15 | 3 | 300 | uncoated insulin particles |
| | 4 | 100 | HAX42/PAX2 with 300 iu particle loading |
| | 5 | 300 | HAX42/PAX2 (40mer) particles |
| | 6 | 300 | HAX42 (40mer) particles |
| | 7 | 300 | HAX42 particles + 10-fold excess free HAX42 (40mer) |
| | 8 | 300 | PAX2 (40mer) particles |
| | 9 | 300 | PAX2 freeze-dried (40mer) particles |
| 20 | 10 | 300 | PAX2 scrambled particles III (40mer) |
| | 11 | 300 | PAX2 scrambled particles IV (19mer) |
| | 12 | 300 | 5PAX5/P31 (40mer) particles |
| | 13 | 300 | P31 (40mer) particles |
| | 14 | 300 | 5PAX5 (40mer) particles |
| | 15 | 300 | HAX42 (27mer) particles |
| | 16 | 300 | PAX2 (20mer) particles |
| 25 | 17 | 300 | P31 (20mer) particles |
| | 18 | 300 | PAX2 (15mer) particles |
| | 19 | 300 | P31 (15mer) particles |
| | 20 | 300 | P31 D-form I(5 D-arginine) (16mer) particles |
| | 21 | 300 | P31 D-form II(2 D-arginine) (16mer) particles |
| 30 | 22 | 300 | HAX42 (10mer) |

Availability of insulin following administration was assessed relative to a 1 and 20iu subcutaneous dose because the response to increasing subcutaneous doses of bovine insulin does not increase linearly over the range of 1 to 20iu. Data up to three hours post-dosing was available for most animals.

5 Therefore, availability was first assessed using individual AUC(0-3h) data estimated from baseline-subtracted data for which data up to 3 hours was available. This approach may lead to an underestimation of the availability as some animals that gave a high response often did not survive for 3 hours and, therefore, were excluded from the analyses. In an attempt to
10 capture as much of these high responses observed at the earlier timepoints as possible, the mean baseline-subtracted plasma concentration data was used to estimate an AUC for each group. Table 35 shows the results based on this second approach (AUC(0-3h) calculated from the mean plasma concentration data).

15

Table 35

| | Group | Dose iu | Mean AUC _(0-3h) | F vs. 1 iu | F vs. 20 iu |
|----|----------|---------|----------------------------|------------|-------------|
| | 1 | 0 | 2.14 | | |
| | 2a | 1 | 875.27 | 100.00 | 28.86 |
| | 2b | 2 | 2439.36 | 139.35 | 40.22 |
| | 2c | 3 | 3671.44 | 139.82 | 40.36 |
| 20 | 2d | 4 | 6912.18 | 197.43 | 56.98 |
| | 2e | 10 | 27224.41 | 311.04 | 89.77 |
| | 2f | 20 | 60651.28 | 346.47 | 100.00 |
| | 2g | 4 | 14255.49 | 407.17 | 117.52 |
| | 3 | 300 | 10677.78 | 4.07 | 1.17 |
| | 3 -Rat43 | 300 | 4645.06 | 1.77 | 0.51 |
| | 4 | 100 | 3527.18 | 4.03 | 1.16 |
| | 5 | 300 | 27112.26 | 10.33 | 2.98 |
| 25 | 6 | 300 | 33091.68 | 12.60 | 3.64 |
| | 7 | 300 | 9303.09 | 3.54 | 1.02 |
| | 8 | 300 | 34241.83 | 13.04 | 3.76 |
| | 9 | 300 | 10968.83 | 4.18 | 1.21 |
| | 10 | 300 | 27692.78 | 10.55 | 3.04 |
| | 11 | 300 | 3004.29 | 1.14 | 0.33 |
| | 12 | 300 | 18852.61 | 7.18 | 2.07 |
| | 13 | 300 | 20278.43 | 7.72 | 2.23 |
| | 14 | 300 | 17400.38 | 6.63 | 1.91 |
| 30 | 15 | 300 | 16775.69 | 6.39 | 1.84 |
| | 16 | 300 | 14217.47 | 5.41 | 1.56 |
| | 17 | 300 | 8197.97 | 3.12 | 0.90 |

| | | | | |
|----|-----|----------|------|------|
| 18 | 300 | 25050.59 | 9.54 | 2.75 |
| 19 | 300 | 7927.96 | 3.02 | 0.87 |
| 20 | 300 | 21519.57 | 8.20 | 2.37 |
| 21 | 300 | 6322.41 | 2.41 | 0.69 |
| 22 | 300 | 12553.01 | 4.78 | 1.38 |

5 The data for group 3 (uncoated insulin particles) are expressed with and without Rat 43. This animal had an atypically high response to these uncoated particles and, therefore, may have biased the data for this group.

This data shows that a combination of peptide-coated particles (HAX42/PAX2 or 5PAX5/P31) shows no greater
10 availability than particles coated with the individual peptides. Further, peptide-coated particles have a greater availability than uncoated peptides. Scrambling the 40mer PAX2 peptide did not result in a loss of bioavailability. Scrambling the PAX2 peptide and reducing the size to 19mer resulted in a loss of bioavailability although this loss may be
15 attributed in part to the reduction in peptide size. Reducing peptide size resulted in loss of bioavailability. The D-form of P31 (ZElan053, , SEQ ID NO: 319) had increased bioavailability possibly due to greater resistance to peptide breakdown. A competitive excess of peptide resulted in a loss of bioavailability, and freeze drying caused a loss in
20 bioavailability. By way of example, measurement of blood glucose levels showed that the HPT1 and hPEPT1 targeting particles incorporating HAX42, PAX2, P31 (SEQ ID NO: 270), and P31 D-form (ZElan053, , SEQ ID NO: 319) reduced blood glucose levels indicating that the insulin delivered was bioactive.

25 In further studies, insulin was recovered from the targeting particles following particle formation by dissolution and analyzed by electrophoresis in non-denaturing sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The analysis of the insulin by non-denaturing SDS-PAGE and also by western blot transferred to membranes and subsequent screening
30 with an antibody to insulin, indicated that the insulin was

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intact, with no evidence of degradation, dimerization, or aggregation during the process of particle formation.

Study 3

5 An intraduodenal open loop model study was carried
out on Wistar rats (300-350g). Group 1 was administered
leuprolide acetate (12.5 μ g) subcutaneously. Group 2 was
administered intraduodenally uncoated leuprolide acetate
particles (600 μ g, 1.5 ml). Group 3 was intraduodenally
administered leuprolide acetate particles coated with PAX2 (600
10 μ g; 1.5 ml). Group 4 was administered intraduodenally
leuprolide acetate particles coated with P31 (SEQ ID NO:
270) (600 μ g, 1.5 ml). Figure 19 shows the leuprolide plasma
concentration following administration to these four groups.
Both the P31 (SEQ ID NO: 270) and the PAX2 coated
leuprolide particles administered intraduodenally provided
15 enhanced plasma levels of leuprolide relative to subcutaneous
injection.

Homologies of GIT transport-binding peptides to known
proteins are shown in Figures 20, 21A-F, and 22 A-D.

20 The present invention is not to be limited in scope
by the specific embodiments described herein. Indeed, various
modifications of the invention in addition to those described
herein will become apparent to those skilled in the art from
the foregoing description and accompanying figures. Such
modifications are intended to fall within the scope of the
appended claims.

25 Various publications are cited herein, the
disclosures of which are incorporated by reference in their
entireties.

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